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In vitro effects of saponins, tannins and lipids on rumen fermentation and intestinal health markers in a ruminant weaning context

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ABSTRACT

Dairy ruminants often experience physiological disturbances during the post-weaning, including suboptimal rumen fermentation due to an underdeveloped rumen microbiota, and diarrhoea associated with intestinal inflammation and oxidative stress. This study explored six commercial feed additives with a potential dual mode of action to optimise both rumen fermentation and intestinal health in a post-weaning scenario. The experimental treatments included dietary supplementation with saponins (SAP), tannins (TAN), lauric acid (LAU), fish oil (FIO), high-unsaturated olein (HUO) and high-saturated olein (HSO). Additives were tested at four doses (0, 100, 300 and 600 mg/L) with four experimental replicates. Effects on rumen fermentation were evaluated in batch cultures using inocula from dairy lambs and a high concentrate substrate, while intestinal health responses were evaluated in Caco-2 cells. Saponins ($p = 0.01$) and tannins ($p < 0.01$) linearly decreased rumen ammonia-N concentration in batch cultures. In Caco-2 cells, saponins improved metabolic activity ($p = 0.04$), whereas tannins reduced pro-inflammatory IL-6 concentration ($p < 0.01$), suggesting beneficial effects on intestinal health. Lipid sources exerted minimal effects on rumen fermentation and induced intestinal inflammation when supplemented at high doses. Overall, the physiological constrains of dairy ruminants during the post-weaning period, such as an immature rumen microbiota, absence of protozoa and low rumen pH, may limit the efficacy of additives in improving rumen fermentation. Nevertheless, saponins and tannins showed potential to reduce rumen proteolysis and support intestinal health during this critical developmental stage. Further *in vivo* research is required to validate these findings.

HIGHLIGHTS

- Six commercial feed additives were tested to support weaning in artificially reared lambs.
- *In vitro* rumen responses were modest, likely due to immature microbiota and lack of protozoa.
- Tannins and saponins decreased rumen protein breakdown and improved intestinal health.
- The effects of lipids on gut health varied according to lipid type and dose.

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Introduction

In most meat-oriented ruminant production systems, young ruminants remain with their dams, allowing for a progressive rumen microbial colonisation and natural transition from milk to solid feed during the post-weaning period. In contrast, in most intensive dairy farms, newborns are typically separated from their mothers shortly after birth and fed either milk replacer or raw milk. As result, most dairy ruminants reach the weaning period with an immature rumen microbiota and a lack of rumen protozoa due to delayed microbial colonisation. This situation often impairs optimal feed fermentation and consequently reduce dry matter intake (DMI) and animal performance (Belanche et al. 2019). The early transition from milk to solid feed, combined with environmental and social stressors and rapid microbial changes during weaning, has also been linked to alterations in the physiology of the small intestine, resulting in oxidative stress and inflammatory responses (e.g. TNF-α, IL-1β, and IL-6), that predispose young ruminants to gut dysfunctions (Gao et al. 2025). These factors can compromise epithelial barrier integrity, increase intestinal permeability, and facilitate the translocation of luminal antigens or

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pathogens, ultimately triggering diarrhoea during the post-weaning period.

Dietary supplementation with plant secondary compounds (i.e. saponins and tannins) and different types of fatty acids (FA) during the post-weaning period may provide multiple benefits for young dairy ruminants by improving rumen function, intestinal health, and performance. Saponins exert strong anti-protozoal effects in the rumen, shift the volatile fatty acid (VFA) profile towards propionate production, and improve N utilisation efficiency (Rodríguez and Fondevila, 2012). Similarly, tannins, when supplied at moderate levels, form pH-dependent complexes with dietary proteins and carbohydrates (Frutos et al. 2004), thereby modulating microbial fermentation by selectively inhibiting proteolytic bacteria and potentially increasing the flow of undegraded protein to the intestine preventing growth retardation (Frutos et al. 2004; Mezzomo et al. 2011). Dietary supplementation with FA represents an additional energy source during weaning, but it has also been associated with modifications in microbial fermentation and reductions in methane (CH_4) emissions (Fievez et al. 2003; Hess et al. 2008). Medium-chain FA (MCFA, C12:0 to C14:0) possess antimicrobial and anti-protozoal properties and enhance rumen microbial diversity (Hristov et al. 2009; Castro et al. 2022), whereas long-chain FA (LCFA, C13 to C21), particularly monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA), have shown greater efficacy in decreasing rumen CH_4 emissions (Darabighane et al. 2021). Although most of the aforementioned feed additives have shown promising effects when supplemented to adult ruminants, their potential to optimise the post-weaning process remains largely unexplored.

Regarding intestinal health, saponins and tannins possess anti-oxidants and anti-inflammatory properties that may mitigate oxidative stress and reinforce the epithelial barrier function, potentially reducing the incidence of post-weaning diarrhoea (Dai et al. 2023, Molino et al. 2023). Likewise, MCFA serve as energy source for enterocytes, supporting intestinal integrity and barrier function (Zhao et al. 2021), whereas unsaturated LCFA can exert anti-inflammatory effects by down-regulating pro-inflammatory cytokines. (Melendez et al. 2022). However, the effects of these additives on the gut health are highly variable and dependent on their chemical structure. Consequently, identifying the most effective feed additives for artificially reared young ruminants remains challenging, as ideal candidates should have a dual mode of action supporting both rumen function and intestinal health simultaneously.

In vitro models can provide a practical, ethical, and cost-effective approach to investigate feed additives and elucidate their mode of action before animal trials. Batch cultures are widely employed to evaluate the effects of feed additives on rumen fermentation (Durmic et al. 2025), while Caco-2 epithelial monolayers represent the gold standard to screen effects on intestinal barrier function, oxidative stress and inflammatory responses (Śliżewska et al. 2021; Agustinho et al. 2023).

This study aimed to screen six selected commercially feed additives, including saponins, tannins and four lipid sources, as potential candidates to optimise the weaning process in artificially reared ruminants. We hypothesised that some of these additives might exert a dual mode of action improving by improving both rumen fermentation and intestinal health. To address this, two complementary *in vitro* approach were used: i) a dose-response batch culture incubation using rumen inoculum from artificially reared lambs fed a high-concentrate diet, to assess potential improvements in rumen microbial fermentation (i.e. increased gas production and VFA or lower ammonia concentrations), and ii) Caco-2 cell monolayers to evaluate potential positive effects on intestinal health (i.e. increased hindgut cell viability and reduced inflammation and oxidative stress).

Material and methods

Natural feed additives

The following six natural and commercially-available feed additives were selected as they could potentially have positive effects on rumen fermentation and hindgut health: 1) A saponin extract (**SAP)** containing 200 g/kg of extractable material in ethanol (Silvafeed MultiSap®, Silvateam, San Michele Mondovì CN, Italy), obtained from yucca, quillaja and oats; 2) A tannin extract (**TAN)** containing 750 g/kg of tannins, of which 55% were hydrolysable from chestnut and 45% were condensed tannins from quebracho (Silvafeed ByPro®, Silvateam, San Michele Mondovì CN, Italy); 3) Lauric acid (**LAU)** extracted from palm kernel oil (> 990 g/kg, Andrés Pintaluba SA, Reus, Spain); 4) A fish oil extract (**FIO)** obtained from halibut liver (NAT® Cofathim, SARL., Vauvillers, France) containing total lipids (307 g/kg), omega-3 FA (153 g/kg), eicosapentenoic acid (41 g/kg) and docosahexaenoic acid (31 g/kg); 5) A high unsaturated olein (**HUO)** containing: PUFA 220–350 g/kg (mostly linoleic acid), SFA 110–190 g/kg, unsaturation level 1.07-1.22 (AL®, Riosa S.A., Jaen, Spain); 6) A high saturated olein (**HSO)** containing: SFA 370–450 g/kg (mostly palmitic and stearic acids), PUFA

160–190 g/kg, unsaturation level 0.62-0.75 (BMV®, Riosa S.A., Jaen, Spain).

All additives were supplied as a solid form and tested at increasing concentrations (0, 100, 300, and 600 mg/L) in two complementary *in vitro* experiments. These doses were selected according to the manufacturers' recommendations, with adjustments made to account for dilution in the *in vitro* models.

In vitro rumen fermentation

A batch culture incubation was performed to evaluate the effects of the additives on rumen fermentation mimicking a post-weaning scenario. Animal procedures were carried out under the Project Licence PI22/25 approved by the Ethic Committee for Animal Experimentation from eh University of Zaragoza, Spain. Rumen fluid was obtained from four Assaf lambs (8 weeks old) reared on a commercial dairy farm (Santa Eulalia, Spain). Lambs were artificially reared with *ad libitum* access to milk replacer, concentrate feed, and barley straw until weaning at seven weeks of age. One-week post-weaning, lambs were slaughtered at a commercial abattoir (Cella, Spain), and rumen contents were collected immediately after evisceration. The rumen fluid from each lamb ($n = 4$) was transported to the laboratory in four airtight thermos flasks, each filtered through two layers of muslin, and diluted (4:1) with an anaerobic incubation buffer adjusted to pH 6.0 to mimic rumen environmental conditions in post-weaned lambs.

Batch culture incubations were conducted in 120 mL Wheaton bottles containing 50 mL of buffered rumen inoculum and 500 mg of a standardised high-concentrate substrate (Table 1). The experimental design included six additives (SAP, TAN, LAU, FIO, HUO and HSO), four doses (0, 100, 300, and 600 mg/L) and four experimental replicates (rumen fluid from four lambs). Additional bottles without substrate or additives were included as blanks. Bottles were incubated anaerobically at 39 °C for 24 h. Gas production (GP) was measured at 3, 6, 12, and 24 h using a manometer (Delta Ohm, Caselle di Selvazzano, Italy) and pressure readings were converted to volume using the ideal gas law with atmospheric pressure corrections. The GP speed rate was calculated as the ratio of hourly GP during the 0–3 h interval to the hourly GP during the 12–24 h interval. Gas samples (4.5 mL) were collected at 3, 10, and 24 h for CH_4 analysis using gas chromatography (Agilent 6890 Series GC System, Santa Clara, USA). After 24 h incubation, bottles were opened, pH was recorded using a CRISON micro-pH metre 2001 (Barcelona, Spain), and two 1 mL samples were collected: one was mixed with 0.25 mL of H_3PO_4 buffer (0.5 mol/L) containing 4–methyl valeric acid as internal standard (2 g/L) for VFA analysis, and the other was mixed with 0.5 mL of HCl (3 mol/L) for ammonia-N determination. Concentrations of rumen protozoa in the initial inocula were assessed using an optical microscope. Methane and VFA concentrations were measured by gas chromatography (Agilent 6890 Series GC System, Santa Clara, USA). Branched-chain volatile fatty acids (BCVFA) was calculated as the sum of iso-butyrate and iso-valerate. The concentration of ammonia-N was determined colorimetrically (Chaney and Marbach, 1962).

Table 1. Ingredients and chemical composition of the experimental diet.

	% in DM
Ingredients	
Barley	30
Wheat	20
Corn	20
Soybean meal	10
Wheat bran	10
Sunflower seeds	5
Distillers	5
Barley straw	8
Chemical composition	
Organic matter	96.6
Crude protein	16.2
Ether extract	2.85
Neutral detergent fibre	23.1
Acid detergent fibre	9.60
Acid detergent lignin	1.48

Caco-2 cell culture

The effects of the feed additives on intestinal health indicators was conducted using the human colon adenocarcinoma cell line Caco-2 (86010202, ECACC, Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained in T75 culture flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 ml/L foetal bovine serum, 2 mmol/L of L-glutamine, 10 mL/L non-essential amino acids, 100 U/mL penicillin and 100 μg/mL streptomycin. Cultures were incubated at 37 °C under a humidified atmosphere containing 5% CO_2. Cells were passaged upon reaching 80% confluence, and four independent experimental runs were performed across consecutive passages (between passages 15 and 19) to ensure experimental repeatability. For each run (considered as an experimental unit), cells were detached using a 0.25% Trypsin-EDTA solution and seeded at a density of 45,000 cells/cm^2 in 96–well plates (0.32 cm^2/well) to facilitate monolayer formation.

Seven days after seeding, cell monolayers were incubated for 24 h in 200 µL of culture medium containing the feed additives. The six additives were initially diluted in pure Dimethyl sulfoxide (DMSO, 1:2 and 1:3) before being added to the cell culture medium at 2% of the total volume, achieving a final concentration of 0, 100, 300, and 600 mg/L. Following incubation, the supernatant was collected and stored at −20 °C for subsequent quantification of malondialdehyde (MDA) and interleukin-6 (IL-6) using a colorimetric assay (RayBiotech, Peachtree Corners, USA) and an ELISA kit (IL-6, Elabscience, Texas, USA), respectively.

Cell metabolic activity (or MTT activity) was assessed *via* mitochondrial dehydrogenase activity using the MTT colorimetric assay. After removing the supernatant, monolayers were washed twice with phosphate-buffered saline and incubated for 4 h with MTT reagent (500 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) in 200 µL of culture medium per well. Following incubation, the supernatant was discarded, and the formazan crystals formed were solubilised with DMSO (200 µL/well). Absorbance was recorded using a Synergy™ HT plate reader (BioTek, Winooski, VT, USA) at 570 nm, with a reference wavelength of 690 nm.

Statistical analyses

All statistical analyses were conducted using SPSS software (IBM Corp., Version 21.0, New York, USA). To determine the minimum effective dose, data for each additive were analysed using the following analysis of variance:

$$Y_{ij} = \mu + D_i + R_j + e_{ij}$$

where Y_{ijk} is the dependent, continuous variable, µ is the overall population mean, D_i is the fixed effect of the dose ($i = 0$ vs 100 vs 300 vs 600 mg/L), A_j is the random effect ($j = 1$ to 4) of the experimental replicate (rumen inoculum or incubation run in experiment 1 and 2, respectively), and e_{ij} is the residual error. When significant effects of doses were detected, means were compared using the LSD test. Additionally, linear (L) and quadratic (Q) orthogonal contrasts were performed to evaluate the dose-response effect for each feed additive. Statistical significance was established at $p < 0.05$, while values between 0.05 and 0.10 were interpreted as indicative of a trend for signification.

Results

Rumen inoculum composition

Microscopic examination confirmed the absence of rumen protozoa in the inoculum from all artificially reared lambs. The initial pH of the rumen inocula averaged 5.84 ± 0,27 total VFA concentration was 107 ± 10.5 mmol/L with proportions of acetate (40.5%), propionate (47.8%) and butyrate (7.4%). These parameters were similar to those observed at the end of the incubation confirming that the *in vitro* conditions were able to mimic the *in vivo* rumen fermentation.

Effects of saponins and tannins

Supplementation with SAP had minimal effects on key rumen fermentation parameters (Table 2). However, SAP supplementation resulted in a linear decrease in ruminal ammonia-N concentration ($p = 0.01$). Furthermore, SAP positively influenced gut health indicators in Caco-2 cell cultures, leading to a linear increase in cell metabolic activity ($p < 0.01$). Supplementing SAP had no effect on MDA concentration, however, the highest SAP dose (600 mg/L) tended to reduce IL-6 concentrations ($p = 0.08$).

Dietary TAN supplementation significantly influenced *in vitro* rumen fermentation and hindgut health parameters (Table 2). Increasing TAN doses linearly reduced ammonia-N concentration ($p < 0.01$) and BCVFA molar proportion ($p = 0.01$). Additionally, TAN moderately suppressed feed fermentation, resulting in a linear decrease in GP ($p = 0.03$) and a linear trend to reduce total VFA concentration ($p = 0.08$) and CH_4 production ($p = 0.08$), while rumen pH and VFA molar proportions remained unchanged. In Caco-2 cell cultures, TAN supplementation significantly reduced IL-6 concentrations across all doses ($p < 0.01$). Furthermore, TAN exerted a quadratic effect on oxidative stress, where the lowest dose (100 mg/L) tended to decrease MDA concentration, while the highest dose tended to increase it. No significant effects on Caco-2 cell metabolic activity were observed with TAN supplementation.

Effects of different types of lipids

Dietary supplementation with LAU had minimal effects on *in vitro* rumen fermentation parameters (Table 3). The only noticeable response was a trend for higher GP speed ratio at the lowest LAU dose ($p = 0.08$). Regarding gut health indicators, LAU supplementation tended to increase cell metabolic activity linearly ($p = 0.09$), but no significant effects were detected on oxidative stress or IL-6 concentration.

Supplementation with FIO showed negligible effects on rumen fermentation. The only observed response was a trend to an increased GP speed ratio ($p = 0.07$). FIO supplementation had differential effects

Table 2. Dose-response effects of saponins (SAP) and tannins (TAN) on *in vitro* rumen fermentation using batch cultures and intestinal health indicators using Caco-2 cells.

	Dose, mg/L							
	0	100	300	600	SEM	P-value	Linear	Quadratic
SAP								
Rumen fermentation								
pH	5.68	5.71	5.71	5.71	0.026	0.82	0.63	0.55
Ammonia-N, mg/L	312[b]	317[b]	311[b]	296[a]	4.42	0.04	0.01	0.18
Total VFA, mmol/L	139	139	134	140	8.47	0.88	0.90	0.49
Acetate, %	38.2	38.4	39.0	37.7	1.03	0.85	0.73	0.45
Propionate, %	43.7	43.2	42.8	43.8	0.713	0.73	0.84	0.29
Butyrate, %	11.6	12.0	11.9	12.1	0.209	0.46	0.23	0.74
Valerate, %	4.40	4.20	4.15	4.18	0.162	0.70	0.44	0.45
BCVFA, %	2.07	2.24	2.17	2.22	0.064	0.28	0.26	0.53
Total GP, ml/d	103	103	103	102	0.870	0.81	0.67	0.41
GP speed ratio	11.2	12.3	11.9	11.7	0.541	0.56	0.87	0.39
CH_4, %	2.60	2.68	2.67	2.77	0.083	0.54	0.19	0.98
Total CH_4, mL/d	2.33	2.36	2.38	2.46	0.090	0.77	0.32	0.91
Intestinal health								
Cell activity, %	100[a]	107[a]	111[ab]	120[b]	4.01	0.04	0.01	0.71
MDA, µmol/L	1.08	1.11	1.15	0.700	0.295	0.69	0.34	0.50
IL-6, ng/L	0.190	0.133	0.185	0.111	0.022	0.08	0.09	0.42
TAN								
Rumen fermentation								
pH	5.68	5.71	5.70	5.67	0.031	0.74	0.54	0.43
Ammonia-N, mg/L	312[a]	293[b]	261[c]	227[d]	4.44	<0.01	<0.01	0.14
Total VFA, mmol/L	139	129	130	124	4.59	0.21	0.08	0.69
Acetate, %	38.2	37.9	39.4	40.3	1.07	0.40	0.12	0.98
Propionate, %	43.7	43.5	42.7	42.4	0.604	0.39	0.11	0.72
Butyrate, %	11.6	12.2	11.8	11.7	0.333	0.59	0.66	0.51
Valerate, %	4.40	4.22	4.08	3.98	0.155	0.32	0.09	0.55
BCVFA, %	2.07	2.15	1.95	1.72	0.097	0.06	0.01	0.51
Total GP, mL/d	103	104	102	99.1	1.24	0.11	0.03	0.39
GP speed ratio	11.2	11.8	13.0	12.7	0.682	0.28	0.12	0.25
CH_4, %	2.60	2.78	2.65	2.67	0.078	0.44	0.98	0.63
Total CH_4, mL/d	2.33	2.48	2.31	2.16	0.093	0.19	0.08	0.43
Intestinal health								
Cell activity, %	100	106	97.3	95.7	4.27	0.37	0.22	0.86
MDA, µmol/L	1.08	0.738	0.925	1.60	0.263	0.19	0.09	0.18
IL-6, ng/L	0.190[b]	0.074[a]	0.085[a]	0.089[a]	0.014	<0.01	<0.01	<0.01

SEM, standard error of the mean.
[a-d]Means with different superscript differ ($n = 4$).

on gut health indicators as moderate doses (100 to 300 mg/L) tended to quadratically improve cell metabolic activity ($p = 0.03$) and reduce oxidative stress indicator in Caco-2 cells ($p = 0.09$), while the highest dose (600 mg/L) exhibited the opposite effects. Increasing FIO supplementation did not affect IL-6 concentrations.

Dietary supplementation with HUO had minimal effects on *in vitro* rumen fermentation (Table 4) but it tended to linearly increase the GP speed ratio ($p = 0.06$). A quadratic decrease in GP was detected when HUO was supplemented at 100 and 300 mg/L ($p = 0.01$). Supplementation with HUO at 300 mg/L tended to quadratically increase Caco-2 cell metabolic activity ($p = 0.02$), moreover HUO tended to linearly increase IL-6 concentration ($p = 0.09$).

Supplementation with HSO also had limited effects on rumen fermentation. However, increasing HSO levels tended to linearly shift fermentation pattern towards higher butyrate ($p = 0.07$) in detriment to propionate ($p = 0.09$), and without affecting total VFA concentration. Notably, HSO supplementation linearly enhanced Caco-2 cell metabolic activity ($p = 0.04$) but had no significant effects on oxidative stress or IL-6 concentration.

Discussion

Experimental approach

Although the present study relied on *in vitro* models, we acknowledge that their direct extrapolation to animal performance and physiology must be interpreted with caution for several reasons. Batch culture incubations are among the most widely used techniques for screening the effects of feed additives due to their simplicity, repeatability, and low cost. However, a recent guideline on the use of *in vitro* systems for evaluating feed additives in ruminants identified two major limitations (Durmic et al. 2025). First, the short

Table 3. Effects of lauric acid (LAU) and fish oil (FIO) on *in vitro* rumen fermentation using batch cultures and intestinal health indicators using Caco-2 cells.

	Dose, mg/L							
	0	100	300	600	SEM	P-value	Linear	Quadratic
LAU								
Rumen fermentation								
pH	5.68	5.71	5.70	5.71	0.026	0.84	0.51	0.77
Ammonia-N, mg/L	312	322	316	310	11.6	0.88	0.70	0.67
Total VFA, mmol/L	139	136	149	144	9.96	0.80	0.56	0.62
Acetate, %	38.2	39.4	38.2	38.5	0.563	0.41	0.71	0.87
Propionate, %	43.7	42.6	43.4	43.2	0.373	0.27	0.97	0.50
Butyrate, %	11.6	11.7	12.1	12.0	0.204	0.43	0.23	0.31
Valerate, %	4.40	4.11	4.17	4.13	0.117	0.59	0.25	0.50
BCVFA, %	2.07	2.15	2.20	2.21	0.077	0.55	0.24	0.47
Total GP, mL/d	103	102	102	101	1.36	0.79	0.42	0.95
GP speed ratio	11.2	13.2	11.7	12.7	0.497	0.08	0.32	0.80
CH_4, %	2.60	2.53	2.66	2.57	0.087	0.76	0.89	0.61
Total CH_4, mL/d)	2.33	2.19	2.37	2.24	0.111	0.67	0.89	0.74
Intestinal health								
MTT activity, %	100	100	97.3	112	4.94	0.20	0.09	0.19
MDA, µmol/L	1.08	0.925	0.963	0.925	0.162	0.88	0.62	0.75
IL-6, ng/L	0.190	0.170	0.154	0.142	0.042	0.83	0.39	0.78
FIO								
Rumen fermentation								
pH	5.68	5.75	5.74	5.74	0.028	0.40	0.35	0.34
Ammonia-N, mg/L	312	312	309	312	3.39	0.83	0.97	0.43
Total VFA, mmol/L	139	141	137	133	8.44	0.92	0.56	0.87
Acetate, %	38.2	37.8	38.6	38.8	0.701	0.78	0.41	0.96
Propionate, %	43.7	43.7	43.4	43.1	0.416	0.73	0.29	0.92
Butyrate, %	11.6	11.9	11.7	11.8	0.244	0.89	0.91	0.80
Valerate, %	4.40	4.28	4.09	4.16	0.129	0.40	0.20	0.27
BCVFA, %	2.07	2.26	2.18	2.15	0.082	0.45	0.87	0.33
Total GP, mL/d	103	101	100	102	1.80	0.66	0.99	0.23
GP speed ratio	11.2	12.4	12.9	12.7	0.410	0.07	0.05	0.07
CH_4, %	2.60	2.56	2.53	2.55	0.071	0.93	0.67	0.63
Total CH_4, mL/d)	2.33	2.19	2.17	2.21	0.104	0.71	0.55	0.38
Intestinal health								
Cell activity, %	100	110	115	96.4	5.37	0.12	0.44	0.03
MDA, µmol/L	1.08	0.925	0.963	1.79	0.302	0.22	0.09	0.22
IL-6, ng/L	0.190	0.121	0.138	0.116	0.025	0.21	0.15	0.43

SEM, standard error of the mean ($n = 4$).

incubation time (24 h) prevents assessment of the adaptation of the rumen microbiota to the additive and the persistence of its effects. Second, the use of highly buffered media helps prevent acidification caused by the accumulation of fermentation products but may bias results if pH values differ from those observed *in vivo*. Additional factors, including the type of donor animals, which influences rumen physiology (e.g. rumination, saliva production, dilution rate), the diet fed to donors, which shapes microbial communities, and the choice of incubation substrate, can also affect outcomes (Yáñez-Ruiz et al. 2016).

Our study was designed to simulate post-weaning conditions in artificially reared ruminants; therefore, rumen inocula were obtained from artificially reared lambs fed a high-concentrate diet. As expected, these inocula lacked rumen protozoa, a common feature in non-mothered lambs. This is relevant because protozoa contribute to fibre degradation and CH_4 production (Newbold et al. 2015). In previous work, we also demonstrated that artificially reared lambs exhibit lower bacterial diversity and reduced rumen fermentative activity at weaning compared with mothered lambs (Belanche et al. 2019). The substrate consisted of 92% concentrate feed rich in rapidly fermentable carbohydrates, which resulted in high VFA concentrations (136 ± 6.58 mmol/L), comparable to those observed in weaned lambs (Belanche et al. 2011). Similarly, the buffering capacity of the *in vitro* system was adjusted to achieve an average pH similar to the pH of the inocula and within the range (5.40–6.05) reported in lambs fed diets containing 50–100% concentrate (Belanche et al. 2011). Altogether, the simplified microbiota, absence of protozoa, and low rumen pH may explain the modest effects observed for most tested feed additives on *in vitro* rumen fermentation. Unfortunately, our experimental setup did not allow assessment of microbial shifts, as batch cultures are intended for short incubations that do not support sustained microbial adaptation. These preliminary findings should be validated either using longer-term *in vitro* systems (e.g. Rusitec or dual-flow continuous fermenters) or through *in vivo* studies.

Table 4. Effects of high-unsaturated oleins (HUO) and high-saturated oleins (HSO) on *in vitro* rumen fermentation using batch cultures and intestinal health indicators using Caco-2 cells.

	Dose, mg/L							
	0	100	300	600	SEM	P-value	Linear	Quadratic
HUO								
Rumen fermentation								
pH	5.68	5.71	5.72	5.72	0.026	0.73	0.44	0.52
Ammonia-N, mg/L	312	300	301	301	5.91	0.46	0.37	0.35
Total VFA, mmol/L	139	124	133	114	8.50	0.26	0.12	0.75
Acetate, %	38.2	38.6	38.8	38.5	0.939	0.98	0.85	0.73
Propionate, %	43.7	43.1	43.1	43.1	0.557	0.87	0.61	0.63
Butyrate, %	11.6	12.0	11.9	12.0	0.322	0.50	0.17	0.65
Valerate, %	4.40	4.25	4.12	4.20	0.133	0.55	0.35	0.30
BCVFA, %	2.07	2.10	2.11	2.13	0.081	0.96	0.63	0.84
Total GP, mL/d	103	98.6	98.6	103	1.24	0.06	0.46	0.01
GP speed ratio	11.2	11.8	12.2	12.4	0.352	0.18	0.06	0.35
CH_4, %	2.60	2.43	2.58	2.60	0.073	0.32	0.43	0.55
Total CH_4, mL/d)	2.33	2.08	2.18	2.33	0.107	0.34	0.58	0.18
Intestinal health								
Cell activity, %	100	107	118	102	4.95	0.12	0.83	0.02
MDA, µmol/L	1.08	1.11	0.963	0.925	0.216	0.91	0.52	0.92
IL-6, ng/L	0.190	0.110	0.242	0.249	0.042	0.14	0.09	0.98
HSO								
Rumen fermentation								
pH	5.68	5.72	5.72	5.72	0.025	0.67	0.41	0.54
Ammonia-N, mg/L	312	311	313	312	4.69	1.00	0.95	0.93
Total VFA, mmol/L	139	134	136	131	5.92	0.85	0.47	0.98
Acetate, %	38.2	39.4	39.1	38.9	0.365	0.19	0.57	0.12
Propionate, %	43.7	42.6	42.6	42.8	0.307	0.09	0.20	0.06
Butyrate, %	11.6	11.7	11.9	12.0	0.122	0.26	0.07	0.51
Valerate, %	4.40	4.13	4.21	4.17	0.118	0.44	0.40	0.45
BCVFA, %	2.07	2.17	2.17	2.18	0.071	0.67	0.40	0.52
Total GP, mL/d	103	102	101	99.8	1.30	0.48	0.15	0.67
GP speed ratio	11.2	11.9	11.8	12.0	0.400	0.53	0.29	0.65
CH_4, %	2.60	2.51	2.53	2.62	0.081	0.76	0.66	0.40
Total CH_4, mL/d)	2.33	2.16	2.22	2.30	0.112	0.72	0.87	0.40
Intestinal health								
Cell activity, %	100	102	111	120	6.53	0.20	0.04	0.92
MDA, µmol/L	1.08	0.813	0.813	0.850	0.260	0.75	0.58	0.47
IL-6, ng/L	0.190	0.124	0.144	0.173	0.029	0.44	0.91	0.23

SEM, standard error of the mean ($n = 4$).

In relation to intestinal health, despite their human colonic origin, Caco-2 cells have the inherent ability to differentiate and acquire a mature enterocyte phenotype, exhibiting characteristics typical of the small intestine. In culture, Caco-2 cells develop apical–basolateral polarity, form functional tight junctions, display apical microvilli, and possess passive diffusion mechanisms and immune markers. They also express enzymes (e.g. aminopeptidase, lactase, sucrase–isomaltase) and transporters characteristic of small-intestinal enterocytes (Kus et al. 2023). These structural and functional features represent conserved principles of the mammalian small intestine and support the use of Caco-2 cells as a valid mechanistic model to investigate absorption, transport, and metabolic processes, such as inflammation and oxidative stress, relevant to ruminant species.

The main potential limitations of using Caco-2 cells for ruminant studies can be attributed to: 1) species-specific features of ruminant enterocytes that are not fully reproduced by human-derived cells; 2) the oversimplification of the cellular heterogeneity and three-dimensional architecture of the ruminant intestine; and 3) the absence of host–microbiota interactions. To address these limitations, the most physiologically relevant models currently available for studying ruminant intestinal function include ruminant-derived cell lines, 3D intestinal epithelium models, and organoids generated from primary cells (Zhan et al. 2017; Derricott et al. 2019). However, their use remains restricted by several practical constraints: they require fresh ruminant tissue, exhibit substantial inter-individual variability, involve complex and costly culture procedures, and demand highly specialised personnel. For these reasons, well-characterized and reproducible models such as Caco-2 cells continue to be valuable tools for early-stage screening and mechanistic investigations, as intended in our study. In this context, a recent study reported that acidic pH and lipopolysaccharides induced higher levels of pro-inflammatory cytokines and increased tight-junction permeability in Caco-2 cells used as an experimental

model of the weaning transition in dairy calves (Agustinho et al. 2023).

Effects of saponins

Saponins are plant-derived metabolites known to enhance animal productivity, mainly due to their potent anti-protozoal activity in the rumen (Patra and Saxena, 2009). A meta-analysis quantified that defaunation significantly increase BW gain (+4.6%) due to a higher microbial protein flow to the duodenum (+30.4%), which was attributed to a lower protein degradation (−26.2%) (Newbold et al. 2015). The consistent decrease in rumen ammonia-N concentration observed when saponins are supplemented (Bach et al. 2005) has been mostly associated to lower bacterial breakdown by protozoa. Despite the absence of protozoa in the rumen inoculum used in this study, SAP supplementation led to a linear decrease in ammonia-N concentration (from 312 to 296 mg/L), while maintaining a similar BCVFA concentrations. As iso-butyrate and iso-valerate are fermentation products derived from the valine and leucine degradation, this observation suggests that protein degradation remained unchanged, and ammonia-N concentration (over 200 mg/L) were still adequate for supporting the microbial fermentative activity (Nagadi et al. 2000). However, the observed reduction in ammonia-N levels, coupled with unchanged total VFA production suggest an increase in bacterial N uptake, potentially enhancing microbial protein synthesis (Bach et al. 2005). Another hypothesis is the direct inhibition of hyper-ammonia producing bacteria by saponins (Patra and Saxena, 2009) Long-term *in vivo* studies are warranted to validate the persistency of these effects as rumen bacteria can often adapt by developing the ability to inactivate saponins.

Saponins are also known to interact with sterol moieties in mucosa cell membranes, facilitating nutrient absorption and improving animal performance. Moreover, the effectiveness of saponins is highly influenced by their botanical source, dietary conditions, and the physiological conditions of the ruminant (Patra and Saxena, 2009). The SAP extract used in this study consisted of a combination of triterpenoids saponins from *Quillaja Saponaria*, and steroid saponins from *Yucca shidigera* andd *Avena sativa*. In our study SAP supplementation increased up to 20% Caco-2 cellular metabolic activity and tended to linearly decreased IL-6 levels (up to −42%), moreover they numerically decrease MDA values (−36%) when supplemented at 600 mg/L. These beneficial effects of SAP may be attributed to the bioactive compounds present in these plants. *Yucca* is rich in *Sarsasapogenin, Smilagenin* and *Tigogenin*, which exhibit dual antioxidant properties by neutralising reactive oxygen species (ROS) and enhancing endogenous antioxidant enzymes such as superoxide dismutase and glutation peroxidase. They also exert anti-inflammatory effects by downregulating pro-inflammatory cytokines, including IL-6, IL-1β and TNF-α (Dai et al. 2023). Similarly, *Quillaja* is rich in triterpenoid saponins such as quillaic acid and QS-21, which possess anti-oxidant and anti-inflammatory properties (Dai et al. 2023). In addition, oats contain Avenacoside A and B, which, after conversion to desgruco-avenacosde A and B, can also exert anti-oxidant and anti-inflammatory properties (Sharma et al. 2023). These mechanisms align with findings from previous studies, in which lambs supplemented with saponins exhibited lower plasma IL-6 and MDA concentrations (Yang et al. 2023), suggesting that SAP could potentially promote intestinal health during the post-weaning period.

Effects of tannins

Tannins are polyphenolic compounds primarily known for their ability to bind proteins which limits their availability to rumen microbes. Previous studies have demonstrated that both hydrolysable and condensed tannins can reduce N degradation and improve N utilisation efficiency *in vitro* (Fonseca et al. 2023) and *in vivo* (Mezzomo et al. 2011). However, the extent of tannin-protein binding being influenced by factors such as molecular weight, isoelectric point, compatibility of binding sites, protein tertiary structure, digestion kinetics, and diet composition (Fonseca et al. 2023). The TAN extract used in the present study, composed of hydrolysable tannins from and condensed tannins from quebracho, significantly inhibit proteolysis, as evidenced by a linear decrease in ammonia-N concentration (up to −27%) and BCVFA molar proportion (up to −17%). Notably, ammonia-N levels remained above the threshold values required to support microbial growth (Nagadi et al. 2000). These results confirm that tannin-protein complexes remain stable at low ruminal pH (5.6-5.7), being consistent with previous reports showing enhanced intestinal bypass of rumen-undegraded protein in beef cattle fed high-concentrate diets supplemented with tannins (Mezzomo et al. 2011).

Tannins can also directly inhibit extracellular microbial enzymes and various microbes, including bacteria,

fungi, protozoa, and methanogens (Fonseca et al. 2023). This inhibitory effect is associated with reductions in CH_4 production (Jayanegara et al. 2015). In our study, high-dose TAN supplementation (600 mg/L) resulted in a slight decrease in CH_4 production (−7.3%), accompanied by lower total VFA concentrations (−10.8%) and GP (−3.8%) suggesting a possible reduction in substrate fermentation rather than a shift in fermentation pathways. These findings are consistent with previous *in vivo* research reporting CH_4 reductions of up to −26.5% in lambs supplemented with condensed tannins during the post-weaning period (Pathak et al. 2017). However, they also highlight the potential negative effects of high tannin concentrations on rumen fermentation (Frutos et al. 2004). Further investigations including a detailed characterisation of the rumen microbiota are warranted to clarify the effects of TAN on specific microbial populations.

In relation to gut health, tannins can exert antioxidant effects through multiple mechanisms, such as reducing oxidative stress, protecting cellular components (i.e. proteins, lipids, and DNA), and preserving membrane integrity (Molino et al. 2023). This was reflected in our study, where the lowest TAN dose (100 mg/L) resulted in a numerical decrease (−32%) in MDA concentration, suggesting lower oxidative stress. Similar anti-oxidant effects have been reported in calves supplemented with 4 g/d of hydrolysable tannins in milk that improved BW gain, enhanced plasma antioxidant capacity, and reduced both diarrhoea incidence and faecal pathogen shedding compared with un-supplemented diet (Dell'Anno et al. 2024). Our study reported that TAN supplementation did not affect Caco-2 metabolic activity. However, the highest TAN appeared to increase oxidative stress, which may be attributed to either a pro-oxidant effect or inhibition of key detoxification enzymes with high tannin doses.

It has been suggested that the main active compound from chestnut tannins (i.e. vescalagin and castalagin) and from quebracho (i.e. fisetinidin and robinetinidin) have strong anti-oxidant and anti-inflamatory properties (Piazza et al. 2023). These compounds can inhibit key signalling molecules such as prevent inflammatory pathway. This suppression reduces the transcription of pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β. Furthermore, tannins can downregulate Toll-like receptor (TLR) expression, particularly TLR4(Saleh et al. 2021). Our study supports these mechanisms, as TAN supplementation decreased in IL-6 concentrations (53 to 61%) across doses. Similar reductions in pro-inflammatory cytokines (IL-1β and TNF-α) have been reported in lambs supplemented with tannins and fed high-concentrate diets (Lin et al. 2024). These findings suggest that tannins should be further studied *in vivo* as a promising strategy to optimise the weaning process in lambs.

Effect of different types of lipids

In the rumen, dietary lipids undergo rapid and extensive hydrolysis by microbial lipases releasing glycerol and free FA. Glycerol is then metabolised by ruminal microorganisms to produce VFA, while the liberated FA can exert antimicrobial effects, leading to shifts in the molar proportions of VFA (Hidalgo-Hernández et al. 2025). Lipids can also decrease the metabolic activity and population of rumen protozoa, though this effect varies depending on FA chain length and saturation level (Newbold et al. 2015).

Lauric acid, used as a source of MCFA, has been found to exhibit antimicrobial properties against methanogens and certain rumen bacteria (Castro et al. 2022; Hristov et al. 2009. This bacterial inhibition may stem from reduced microbial adhesion to plant material due to lipid coating, direct cytotoxic effects causing membrane destabilisation, suppression of enzymatic activities, and interference with nutrient uptake (Castro et al. 2022; Poothong et al. 2024). Both *in vitro* (Hristov et al. 2009) and *in vivo* (Dohme et al. 2008) studies have reported decreased ruminal propionate proportions following lauric acid supplementation in high-concentrate diets, likely due to modulation of amylolytic microbes responsible for propionate and lactate production (Poothong et al. 2024). Similarly, Debruyne et al. (2018) observed that prenatal and postnatal supplementation of young goats with MCFA (i.e. coconut oil) at inclusion rate of 40 g/kg DM reduced CH_4 emissions by depressing methanogens. However, under our experimental conditions, LAU supplementation did not modify rumen fermentation patterns or CH_4 production.

Supplementation with fish oil, as a source LCFA, has been shown to reduce methanogenesis mainly by directly targeting methanogens rather protozoa as demonstrated in a meta-analysis by Dai and Faciola, (2019). These authors also found that LCFA have a lower impact on CH_4 production compared to MCFA, likely due to their poor solubility in water, limited ability to penetrate microbial membranes, and reduced microbial utilisation or degradation in the rumen. *In vitro* studies using sheep rumen fluid demonstrated that CH_4 inhibition with fish oil became evident only after 48 h of incubation, with effects depending on the dose and FA unsaturation level (Fievez et al. 2003). In contrast to previous reports (Hristov et al.

2004), our study showed that FIO supplementation, as a source of unsaturated LCFA, did not significantly impact rumen fermentation, in terms of VFA, ammonia-N and CH_4, after 24h of incubation. This discrepancy may be due to the lower FIO doses used in our study (3.6 mL/L vs. 10 mL/L), the reduced antimicrobial activity expected in a protozoa-free inocula, and the low NDF content in the diet, which minimised potential fibre digestion disturbances (Fievez et al. 2003).

Oleins, by-products of vegetable oil extraction and fractionation typically derived from olive, soybean, and sunflower oils, are valued in livestock nutrition for their high FA content, natural antioxidants, and appealing odour that enhances palatability. In our study, two types of oleins with different degrees of saturation were tested. Generally, unsaturated FA exert stronger antimicrobial effects than saturated FA, as they reduced rumen protozoa and cellulolytic bacteria, thereby favouring propionate-producing bacteria over acetate producers (Sun et al. 2022). Our findings confirmed that HUO tended to decrease GP (−4.3%), which could be related to reduced feed digestion, although OM degradability was not assessed in this study. However, the PUFA content in this extract appeared insufficient to act as a significant hydrogen acceptor during bio-hydrogenation resulting in similar CH_4 emissions. These results align with Darabighane et al. (2021), who reported no significant effects of PUFA supplementation on rumen fermentation in dairy cows, although a moderate reduction in CH_4 emissions (−4.7%) was observed in the presence of rumen protozoa. A further characterisation of the rumen FA profile could provide more light about the mode of action of this additive.

Our study also showed that dietary supplementation with HSO at concentrations up to 600 mg/L (equivalent to 57 mg saturated lipids/g) had no effect on diet digestion, as GP and total VFA remained stable, but induced a shift from propionate towards butyrate production. Similarly, Matsuba et al. (2019) found no significant effects on rumen fermentation and microbiota when palm oil was supplemented *in vitro* at 50 g/kg, though higher doses (150 g/kg) reduced VFA concentration due to an antimicrobial activity against fibrolytic bacteria and methanogens.

Lipid supplementation in livestock diets significantly influences hindgut health by modulating gut microbial composition, barrier integrity, inflammation, and nutrient absorption (Hess et al. 2008). These effects largely depend on the chain length and degree of saturation of the FA involved: supplementation with MCFA have been shown to enhance tight junction integrity, reduce leaky gut syndrome, improve resilience against stressors, and support epithelial repair (Zhao et al. 2021). Furthermore, MCFA serve as a direct energy source for enterocytes, as they are rapidly absorbed in the small intestine without requiring bile salts, thereby enhancing gut function (Li et al. 2018). Our findings align with these observations, as LAU supplementation tended to increase up to 12% cell metabolic activity when supplemented at 600 mg/L without increasing inflammation or oxidative stress markers in Caco-2 cells. Similarly, Wang et al. (2023) found that LAU supplementation improved immune function in transition dairy cows by reducing the occurrence of inflammatory diseases, likely through inhibition of NF-κB signalling and cytokine production (e.g. TNF-α, IL-6). However, no significant anti-inflammatory effect of LAU was detected in our study.

Dietary supplementation with unsaturated LCFA has also been reported to enhance tight junction integrity, reduce gut permeability, and support epithelial health. Specifically, LCFA-rich omega-3 FAs (e.g. fish oil) are known to reduce inflammation and promote gut homeostasis. Our study demonstrated that FIO supplementation promoted a 15% quadratic increase in Caco-2 metabolic activity when administered at low doses (100–300 mg/L), being also accompanied by a numerical decrease in the oxidative stress indicator. Melendez et al. (2022) also reported that adding fish oil (30 g/d) to milk replacers resulted in higher BW gain during the pre-weaning period, increased DMI during post-weaning, and reduced blood concentrations of inflammatory markers (i.e. haptoglobin, IL-1β, and TNF-α) in dairy calves compared to those supplemented with canola oil. Notably, FIO supplementation did not induce pro-inflammatory effects in Caco-2 cells; however, at a dose of 600 mg/L decreased cell metabolic activity and increased in 65% the oxidative stress marker. Although EPA and DHA have anti-inflammatory properties at low doses, excessive supplementation may negatively impact gut cell metabolic activity and induce oxidative stress (Dasilva et al. 2015). This may occur through increased lipid peroxidation and the generation of ROS, particularly when vitamin E supply is insufficient (Zheng et al. 2024).

The degree of FA saturation is another critical factor influencing the impact of lipid supplementation on intestinal health. Previous *in vitro* studies have shown that polyunsaturated oleins mitigate inflammatory bowel diseases by downregulating pro-inflammatory IL-6 and IL-8, and inhibiting NF-κB in intestinal epithelial cells (Marion-Letellier et al. 2008; Mobraten et al. 2013). Consistent with these findings, our study

demonstrated that HUO, rich in linoleic acid (C18:2 n-6), increased up to 18% Caco-2 cell metabolic activity and reduced 42% IL-6 concentration when supplemented at 300 and 100 mg/L, respectively. However, higher HUO doses led to a 31% increase in IL-6, indicating a pro-inflammatory response. Since HUO did not increase the oxidative stress indicator, the negative effects observed at high doses might be attributed to excessive PUFA incorporation into phospholipid bilayers, compromising cell membrane stability. Furthermore, linoleic acid serves as a precursor to arachidonic acid (C20:4 n-6), which is metabolised *via* the cyclooxygenase and lipoxygenase pathways, leading to the release of pro-inflammatory cytokines (Komprda, 2012).

Contrary to previous reports suggesting that saturated FA supplementation disrupts tight junction proteins, increases intestinal permeability, and upregulates pro-inflammatory signalling pathways (Snodgrass et al. 2013), our findings indicate a different trend. Specifically, HSO supplementation linearly increased Caco-2 metabolic activity by up to 20% without increasing oxidative stress or inflammation markers. While the positive effects of palmitic acid and stearic acid on Caco-2 metabolic activity may seem unexpected, these saturated FAs can serve as an energy source when metabolised *via* β-oxidation, thereby promoting cell proliferation and survival when the energy supply represents a limiting factor (Marion-Letellier et al. 2008).

Overall, our findings suggest that dietary supplementation of young lambs with moderate amounts of FAs had minor effects on the rumen fermentation and gut health indicators. However, it has been suggested that lipid inclusion should be limited to a maximum of 60 g/kg to avoid potential detrimental effects (Toral et al. 2018).

Conclusions

This *in vitro* study allowed the identification of feed additives with the potential to modulate key physiological processes relevant during weaning. Dietary supplementation with saponins from yucca, quillaja and oats or tannins from chesnut and quebracho appears particularly promising due to their dual mode of action: both decreased rumen ammonia-N and improved intestinal health markers (with saponins enhancing cell viability, and tannins reducing IL-6 levels), suggesting that they could plausibly enhance microbial protein utilisation and mitigate post-weaning intestinal inflammation *in vivo*. Likewise, the dose-dependent responses to different lipid sources offer valuable guidance for defining inclusion levels before animal trials. The direct extrapolation of *in vitro* results to *in vivo* implications must be interpreted with caution, as batch cultures and Caco-2 cell assays cannot fully capture the complexity of the rumen or the intestinal environment in young ruminants, including behaviour, digesta dynamics, microbial adaptation, host–microbiota interactions and immune regulation. Therefore, although these *in vitro* results should be considered preliminary, they offer a robust framework for hypothesis generation and justify targeted *in vivo* studies to confirm the magnitude and persistency of these effects under practical production conditions.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

Data are available upon request by contacting the corresponding author.

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