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FINAL REPORT

METHANCUT PROJECT

INALCA

METHANCUT PROJECT

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SUMMARY

The **METHANCUT** project has evaluated the effectiveness of two plant extract-based feed additives (Silvafeed and Anavrin) in reducing enteric methane emissions in beef cattle, adopting an explicitly hypothesis-driven experimental approach.

The main objective was to verify whether the inclusion of additives was capable of **reducing ruminal methanogenesis (primary hypothesis, H1)** and contextually, if this effect could result in **negative interference on production performance, carcass quality and animal welfare (secondary hypothesis, H2).**

The trial was conducted on 36 male Limousine cattle, assigned to three experimental groups (Control, Anavrin, Silvafeed) and raised under standard fattening conditions. Growth performance, feed intake, apparent nutrient digestibility, blood chemistry parameters, carcass and meat characteristics, as well as numerous ruminal health indicators, including macroscopic, histological and histometric measurements, were monitored.

Methane emissions were measured using a Laser Methane Detector, distinguishing between respiratory and eructation emissions, then analysed using mixed linear models supplemented by an approach based on compatibility intervals.

The results indicate that the primary hypothesis (H1) can be accepted. In particular, Silvafeed resulted in a significant reduction in total and respiratory methane emissions, close to 18–20%, supported by compatibility intervals that did not overlap with those of the Control group. Anavrin showed a more modest reduction (approximately 7–8%), but one that is biologically plausible and consistent with the hypothesised mechanisms. The additives acted mainly on the intensity of emissions, without altering the frequency of respiration and eructation events, indicating a direct effect on ruminal fermentation processes.

As for the secondary hypothesis (H2), the results allow it to be rejected. No negative interference was found on the average daily gain, dry matter intake, slaughter yields, meat quality characteristics or the main blood chemistry parameters, which remained within physiological ranges. On the contrary, morphological and histological analyses of the rumen suggest a possible protective effect of the additives on the integrity of the ruminal mucosa, with a reduction in the alterations compatible with parakeratosis and inflammatory infiltration observed in the Control group.

Overall, the confirmation of H1 jointly with the rejection of H2 demonstrate that the additives tested, in particular Silvafeed, can contribute effectively and in a biologically sustainable manner to the mitigation of enteric methane emissions in beef cattle, without compromising production performance, carcass quality or animal welfare.

These results are particularly significant as they were obtained in a food system already characterised by low baseline emissions, reinforcing the biological and practical validity of the findings.

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1 FOREWORD

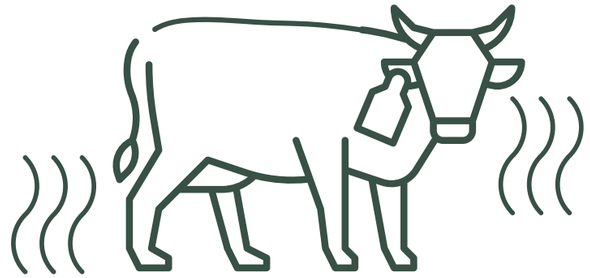
Methane (CH_4) is recognised as one of the main anthropogenic climate-altering gases and, after carbon dioxide (CO_2), represents one of the most significant components of global warming. Unlike CO_2, methane has a higher short-term warming potential, making it a priority target for climate mitigation strategies.

A significant portion of anthropogenic methane emissions is attributable to the agricultural sector and, in particular, ruminants. In these animals, CH_4 originates primarily from ruminal fermentation processes, as a byproduct of the microbial degradation of structural and non-structural carbohydrates, and is subsequently eliminated through eructation and respiration. Enteric methane production is therefore a physiologically inevitable phenomenon, but biologically modulable, as it depends on the dynamics of ruminal fermentation and the composition of the diet.

In the context of European climate transition policies, reducing enteric methane emissions in ruminants is considered a strategic priority, as it allows for direct intervention on one of the main sources of anthropogenic methane. This leads to interest in nutritional solutions capable of modifying the rumen ecosystem and reducing methanogenesis without compromising production performance and animal welfare.

Therefore, **for the European Union, reducing enteric methane emissions represents one of the most important high-priority challenges** in achieving climate neutrality by 2050.

MAIN SOURCES OF METHANE EMISSIONS IN A BEEF FARM:



RUMINAL FERMENTATION AND EMISSIONS FROM MANURE MANAGEMENT

Among the various strategies for reducing enteric methane emissions is the **use of feed additives** that affect the ruminal fermentation process.

2 EXPERIMENTAL HYPOTHESIS

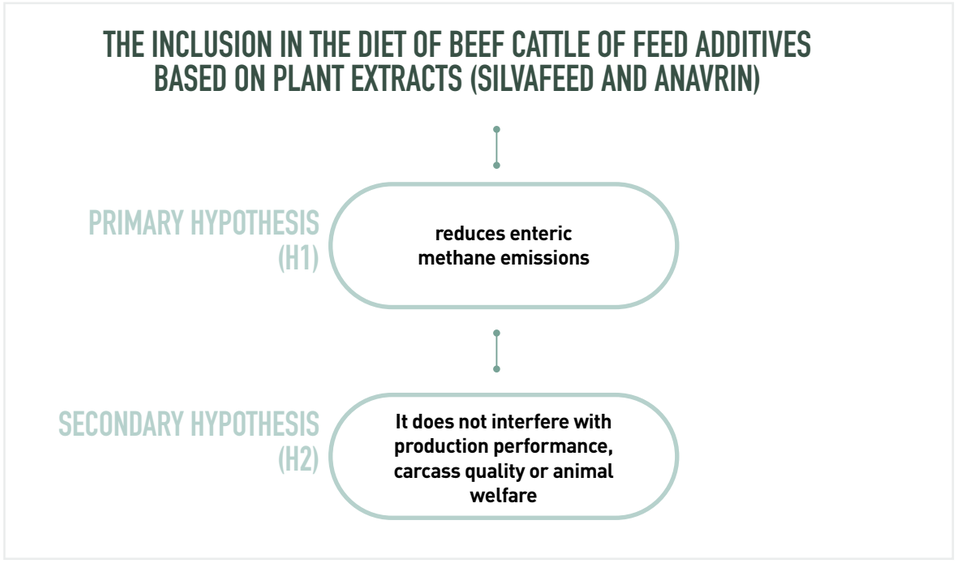
The study was designed and conducted according to an explicitly hypothesis-driven approach, in line with the epistemological and methodological principles outlined by Pulina (2025), according to which an experimental hypothesis must be formulated a priori, be biologically plausible, falsifiable and consistent with the experimental design, clearly distinguishing itself both from mere declarations of intent and from post hoc rationalisations of the results.

Based on available knowledge of the mechanisms **modulating ruminal fermentation exerted by tannins, saponins, essential oils, and bioflavonoids**, as well as preliminary evidence regarding their potential role in **mitigating enteric methanogenesis**, the following primary hypothesis (H1) was formulated: the inclusion of feed additives based on **plant extracts (Silvafeed and Anavrin)** in the diet of beef cattle reduces enteric methane emissions compared to a control diet without additives, through modulation of ruminal fermentation processes. The corresponding null hypothesis (H0) assumes the absence of differences in methane emissions between the experimental groups.

Alongside the primary hypothesis, and in line with the principle of "modesty" and "conservatism" of the scientific hypothesis recalled by Pulina (2025), a secondary hypothesis (H2) was formulated, necessary to verify that any reduction in emissions **is not achieved at the expense of production performance, carcass quality or animal welfare**. In other words, the aim was to verify whether the inclusion of the additives Silvafeed and Anavrin in the diet of beef cattle negatively interferes with weight gain, feed intake, slaughter yields and carcass quality, and may alter the main biological parameters indicative of animal welfare, including metabolic state, rumen integrity and functionality and blood chemistry test indicators. Also in this case, the null hypothesis (H0) postulates the absence of differences between the experimental groups for the production, slaughter and biological parameters considered.

The explicit distinction between primary and secondary hypotheses reflects the need, emphasized by Pulina (2025), to maintain a clear interpretative hierarchy between the main expected effects and the conditions of potential biological interference, avoiding both the risk of "fishing expeditions" and overinterpretations based on marginal results or those inconsistent with the experimental design.

In this context, confirmation of the primary hypothesis (H1) combined with rejection of the secondary hypothesis (H2) would represent a biologically relevant and methodologically sound demonstration of the effectiveness of additives in contributing to the mitigation of enteric methane emissions without compromising the production performance and welfare of beef cattle.



3 MATERIALS AND METHODS

The experimental trial was conducted from January to May 2025, at the Galvana stable (Inalca S.p.A.), in full compliance with the European guidelines on the protection of animals for experimental purposes (ARRIVE guidelines; U.K. Animals -Scientific Procedures- Act, 1986; EU Directive 2010/63/EU).

3.1 ANIMALS AND DIETARY TREATMENTS

Thirty-six male Limousin cattle, homogeneous in weight and age (age: 11.5 ± 2.02 months), were selected from a larger group of 56 animals and assigned to three experimental groups: **Control, Anavrin, and Silvafeed.**

All animals were in good health. Health status was assessed at the start of the trial and monitored throughout the fattening cycle by veterinarians.

The animals were housed in 9 pens, each housing 4 animals (floor area of 5 m^2 per animal) of the same weight.

All animals received the same basal diet, the composition of which is shown in Table 1, supplemented with a commercial concentrate, the composition and nutritional characteristics of which are also shown in the table. For the treated groups (Anavrin and Silvafeed), the concentrate included the additive in the form of meal.

Feed was distributed once a day, in the morning, using a mixer wagon. Details of the diets fed to the three groups are provided below:

GROUP 1 CONTROL
Basal diet formulated to meet nutritional requirements (NASEM, 2016), supplemented with 1.2 kg/head/day of a commercial concentrate

GROUP 2 ANAVRIN
Basal diet supplemented with 1.2 kg/head/day of commercial concentrate containing the additive Anavrin (equivalent to 5 g/100 kg of live body weight)

GROUP 3 SILVAFEED
Basal diet supplemented with 1.2 kg/head/day of commercial concentrate containing the additive Silvafeed (equivalent to 30 g/head)

Table 1 - Composition of the feed ration

	kg/head/day*		% of dry matter
BASAL DIET		**COMPOSITION**	
STRAW	0.60	DRY MATTER	69.78
SUPPLEMENT	0.45	FEED UNIT FOR MEAT	1.07
SOYBEAN MEAL	1.10	CRUDE PROTEIN	12.65
CORN MEAL	3.00	SUGARS	5.19
HYDROGENATED FATS	0.22	STARCH	39.33
BISCUITS	1.60	NDF	24.23
CORN MASH	3.00	FAT	5.73
WHEAT	3.00	TOTAL CA	0.66
COMMERCIAL CONCENTRATE	1.20	TOTAL P	0.40

* Values expressed on an as-fed basis

3.2 IN VIVO MEASUREMENTS AND ANALYSIS

FEED INTAKE AND ANIMAL PERFORMANCE

Feed intake was quantified daily, per pen, recording the amounts fed to the feeder and the resulting residues, collected after 24 hours.

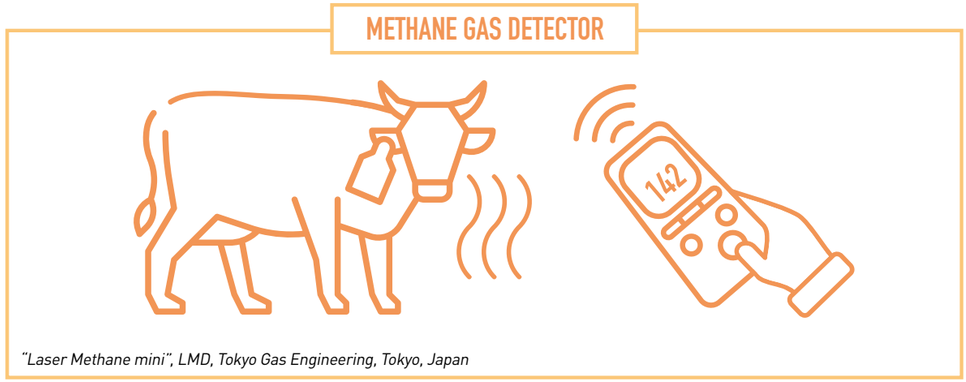
Feed and residue samples were analysed by NIR spectroscopy (TANGO FT-NIR; Bruker Optics GmbH, Ettlingen, Germany) to determine their chemical composition.

To evaluate animal performance, each animal was weighed individually at the beginning of the trial (d0), at the midpoint (d68), and at slaughter (d124). The collected data were used to calculate the average daily gain (ADG).

METHANE MEASUREMENTS

Methane measurements were performed weekly, once in the morning and once in the afternoon, using two laser methane detectors (Methane Gas Detector "Laser Methane mini", LMD, Tokyo Gas Engineering, Tokyo, Japan). Each measurement was recorded continuously every 0.5 seconds for 3 minutes, per animal. Since the LMD measures the concentration of CH_4 in exhaled air, the animal's respiratory cycle was considered.

Given that methane can be released either through eructation, directly from the rumen, or through respiration, following absorption of CH_4 from the rumen or lower digestive tract (Murray et al., 1976), a bimodal distribution of the data was assumed to distinguish CH_4 emissions associated respectively with respiration and eructation. Each measurement taken with the LMD generated a file containing approximately 400 records. The time series of CH_4 values thus obtained was called a "profile" (Figure 1). The profiles were characterised by peaks and valleys; peaks attributable to eructation were generally much higher than those associated with respiration. Before analysis, some signals considered "physiologically implausible" were removed: in particular, values > 100 ppm were eliminated if there were values equal to 0 in both the previous and subsequent records.



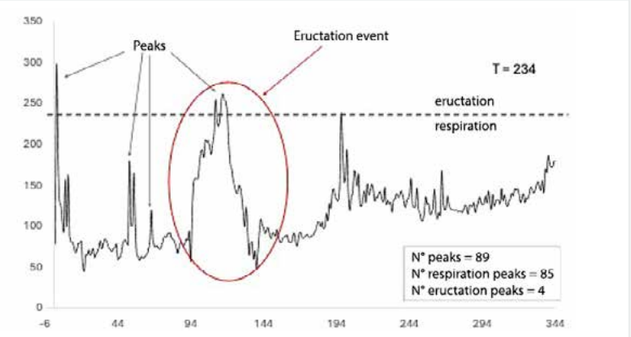
"Laser Methane mini", LMD, Tokyo Gas Engineering, Tokyo, Japan

Peak identification in the CH_4 profiles was performed according to the procedure described by Sorg et al. (2018). For each profile, the difference between a given value (X) and the previous value (X–1) and the difference between X and the value (X–2) were calculated. If the first difference (X–X–1) was < 0 and the second (X–X–2) ≥ 0, the value X–1 was classified as a peak. The distinction between peaks due to eructation and those due to respiration was also performed according to the procedure proposed by Sorg et al. (2018), identifying the threshold (T) as:

$$T = Q3 + [1.5 \times (Q3 - Q1)]$$

where Q1 and Q3 represent, respectively, the first and third quartiles of the distribution of CH_4 peaks in each profile. The phenotypes related to methane emissions were: the means of all CH_4 values, the means of all identified peaks, the means of respiration peaks and the means of eructation peaks, and the number of peaks.

Figure 1 - Example of a measurement profile



EVALUATION OF APPARENT DIGESTIBILITY

The assessment of apparent digestibility (aTTD) was carried out weekly, for the entire duration of the trial. Both the characteristics of the unifeed and the faeces were therefore evaluated using Polispec NIR (Polispec, ITPhotonics, Via Astico, 39. 36030 Fara Vicentino (VI) Italy). Specifically, the assessment of the unifeed was carried out in the morning, when the fresh feed was unloaded into the feeder and the required additive integrations provided for. The measurements were carried out along the entire feeder, dividing it into equal parts corresponding to the different study groups and therefore carrying out 3 measurements per study group (9 measurements in total). These individual measurements were then combined and the daily average was evaluated.

Concomitantly, unifeed samples were also collected to be stored and subsequently analysed by NIR spectroscopy (TANGO FT-NIR; Bruker Optics GmbH, Ettlingen, Germany) and, when necessary, by classical chemistry methods.

Faecal analyses were performed weekly before morning feed administration, collecting a representative pool of faeces from each pen involved in the trial (3 pens per group, 9 pens total). Fresh samples (at the time of defecation) were collected from at least 3 animals per pen, and a pool was then created to allow three weekly measurements per group. Both faecal and mixed feed samples were analysed for dry matter (DM), ash, crude protein (CP), fat, Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), Acid Detergent Lignin (ADL), Acid-Insoluble Ash (AIA), and starch.

ADL and AIA values were used as indigestible internal markers to assess digestibility using the following formula:

$$aTTD\ \% = \frac{\left(\frac{xd}{ADLd - AIAd}\right) - \left(\frac{xf}{ADLf - AIAf}\right)}{\left(\frac{xd}{ADLd - AIAd}\right)} * 100$$

The faecal pH was also evaluated on each pool of faeces, on the fresh sample and after mixing.

BLOOD SAMPLES AND ANALYSIS

Blood samples were collected from the coccygeal vein on day 0, in the morning, before the distribution of the new unifeed, and at the time of slaughter during jugulation. The samples were collected in vacuum tubes containing coagulation activator (silicate) for serological analyses, lithium heparin for plasma analyses (Vacutest, Kima), and EDTA for the complete blood count (CBC).

The samples containing coagulation activator and lithium heparin were centrifuged at 250 g for 20 min and at 500 g for 10 min (15–20°C), respectively, to obtain serum and plasma (Centrifugette 4203, ALC International S.r.l), then stored at –80°C until analysis. The EDTA samples, kept at 4°C after collection, were analysed within 4 hours for the complete blood count. A clinical autoanalyzer (ILAB-650, Instrumentation Laboratory) was used for blood chemistry analyses, following the procedures described by Calamari et al. (2016).

Calibration was performed using commercial standards. For each parameter, in each sample, four different quality controls were applied to verify repeatability and precision.

3.3 SLAUGHTER AND POST-MORTEM MEASUREMENTS

The animals were slaughtered at the Inalca S.p.A. slaughterhouse in Castelvetro di Modena (MO) upon reaching an average weight of 600.4±47.6 kg and after mechanical stunning.

CARCASS PERFORMANCE, PH AND COLOUR

Carcass weight was recorded immediately after slaughter (hot carcass weight), and carcass yield was calculated as a percentage of the hot carcass weight compared to the final live weight. Subsequently, the carcasses were dissected, divided into two halves, and **pH** and colour were measured on the left side.

The pH and temperature measurements were taken 45 minutes and 24 hours after slaughter using a portable pH meter (K21 NWH Inter), with the probe inserted between the 5th and 6th lumbar vertebrae, on the *Longissimus lumborum* muscle.

Muscle and fat colour measurements (*L**, lightness, *a**, redness/greenness, and *b** yellowness/blueness, CIE 1986) were performed using a spectrophotometer (CM-700d/600d, Konica Minolta, Japan). Specifically, measurements were performed following a standardised procedure and with the same illumination for all carcasses. The Minolta colorimeter was set to the *L*, a*, b** system, with illuminate D65, a standard observer at 10°, and an aperture of 8 mm. Before each session, the instrument was calibrated using black and white references. For each sample, the average of five readings was recorded. Measurements were performed 45 minutes after slaughter on the *Rectus abdominis* muscle (between the 6th and 7th rib) and on the subcutaneous fat and 24 hours *post-mortem* on the *Longissimus thoracis* muscle (between the 5th and 6th vertebra of the thoracic region) and on the intramuscular fat.

EVALUATION OF RUMINAL PARAMETERS

At slaughter, all rumens, upon arrival at the tripe plant, were completely opened, and a sample of rumen contents (solid and liquid fractions) was collected from various sites and used for pH determination. The samples were then stored at -80°C for use in determining the microbiota and volatile fatty acids (analysis currently underway). Additionally, a sample of the rumen wall (dorsal sac) was collected, washed, and placed in formalin for histological analysis.

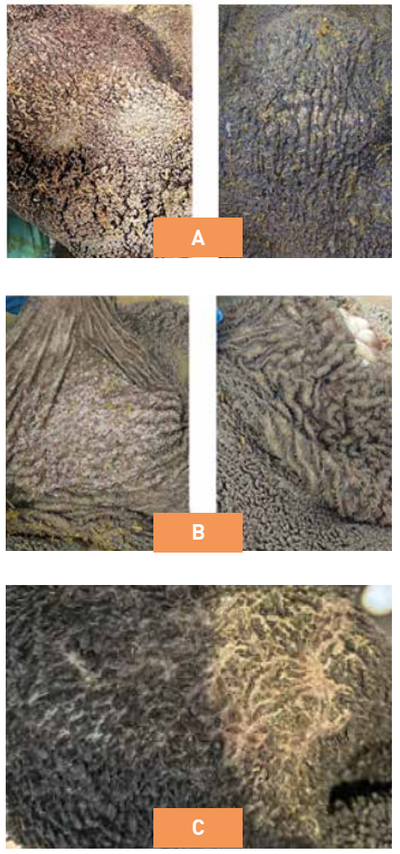
The rumens were inspected in the tripe plant after dissection of the intestines, omasum, and abomasum and following complete emptying and washing by slaughterhouse workers. Specifically, the rumens were assessed and observed for the presence of macroscopic alterations such as hyperkeratosis (Figure 2A), signs of ruminitis (Figure 2B), and star-shaped scars (Figure 2C) following the methodology and references provided by Magrin et al. (2021). The presence/absence of the various indicators was focused on, without evaluating their severity. The presence or absence of rumen parasites of the genus *Paramphistomum* was also recorded. For histological evaluation, rumen wall samples were sectioned using a cryotome and stained with Haematoxylin-Eosin (HE) to evaluate their morphology. The following histometric analyses were performed on the same sections:

- **1** - number of rumen papillae/mm^2;
- **2** - papillae length;
- **3** - papillae width;
- **4** - epithelial thickness;
- **5** - lamina propria thickness;
- **6** - stratum corneum thickness.

MUSCLE SAMPLE COLLECTION

From each *Longissimus thoracis* sample, fragments of approximately 1 cm^3 were taken and frozen in isopentane cooled by liquid nitrogen and stored at -80°C. Sections were prepared for each sample and histochemical analyses were conducted to characterise succinic dehydrogenase activity (an indicator of the type of metabolism, glycolytic or oxidative). This staining allowed the identification of glycolytic, oxidative, and intermediate-type fibres. The number of red/oxidative fibres was assessed and expressed as a percentage.

Figure 2 - Examples used for the visual assessment of rumens proposed by Magrin et al. (2021)



EXAMPLE OF RUMEN WALLS WITH SIGNS OF **HYPERKERATOSIS**

EXAMPLE OF RUMINAL WALLS WITH SIGNS OF **RUMINITIS**

EXAMPLE OF **STAR SCAR**

3.4 STATISTICAL ANALYSIS

Data on initial body weight, body weight at slaughter, hot carcass weight, hot carcass yield, colour, pH and temperature were analysed using the following statistical model:

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

Where Y_{ij} is the dependent variable, μ is the overall mean, T_i is the treatment effect, and e_{ij} is the residual error.

Growth data were analysed using a mixed repeated measures model using the PROC MIXED procedure in SAS.:

$$Y_{ijklm} = \mu + T_i + R_j + (T*R)_{ij} + a_k + b_l + e_{ijklm}$$

Where Y_{ijklm} is the dependent variable, μ is the overall mean, T_i is the treatment effect, R_j is the relief effect, $(T*R)_{ij}$ is their interaction, a_k is the random effect of the animal, b_l is the random effect of the box, while e_{ijklm} is the residual error.

Methane measurement data were initially analysed to determine whether they were normally distributed. Since a non-normal distribution was found, subsequent analyses were performed on both the original values (ppm) and the logarithm-transformed values using the same statistical model used to study growth.

Data were expressed as mean ± SEM (standard error of the mean) with their respective 95% confidence intervals using an approach based on so-called compatibility intervals (Laven and Yang, 2025). Treatment effects were then assessed by considering the width of the confidence intervals, their possible overlap between experimental groups, and the biological consistency of the observed differences with the a priori hypotheses. Given the physiological complexity of methane emissions and the high intrinsic variability associated with these measurements, a technical significance threshold of $P \leq 0.10$ was also adopted, useful for identifying effects consistent with the biological hypotheses formulated, even in the absence of conventional statistical significance. This choice was made explicit a priori and was used exclusively as an interpretative tool, in combination with the analysis of confidence intervals.

Regarding apparent digestibility, statistical analysis was conducted using a mixed linear model (PROC MIXED, SAS) to evaluate the effect of treatment, temporal aggregation (covariate and test period or by month), and their interaction on the digestibility variables. The treatment factor and the aggregation period factor (month or covariate and test period) were treated as fixed effects, while the boxes, considered experimental units, were included as a random effect nested within the group. Since the measurements were repeated over time on the same boxes, the implementation of the repeated measures statistical analysis included a compound symmetry covariance structure to model the correlation between successive observations. Degrees of freedom were corrected using the Kenward–Roger method. For each level of fixed factors, marginal means (LSMeans) were estimated with their respective 95% confidence intervals, obtained from the mixed model. The intervals were calculated based on the standard error estimated by the model and, in multiple comparisons, corrected according to the Tukey–Kramer procedure. The same approach was also used to assess faecal pH.

Haematological data were analysed using JMP pro v19, SAS, and a linear mixed model, considering treatment as a fixed effect and including the covariate for data correction. Variables that did not meet the normality assumption were transformed using the Box–Cox procedure.

Rumen pH was analysed using the PROC MIXED procedure in SAS, including the fixed effect of treatment and the random effect of the individual. Data relating to visual analysis of the rumen were analysed by assessing the difference in frequency distribution within classes using a chi-square test (PROC FREQ).

Finally, regarding histological investigations, data were analysed using a mixed model that considered the fixed effect of treatment and the random effect of the individual.

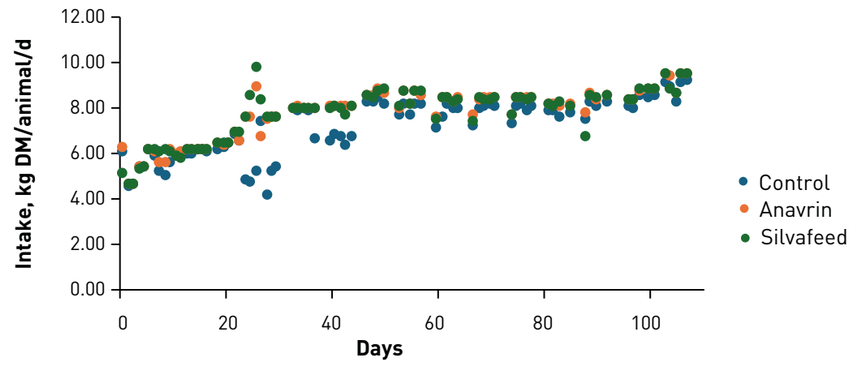
4 RESULTS

4.1 DRY MATTER INTAKE AND GROWTH

Figure 3 shows the results for daily dry matter intake (kg DM/animal/d) of the three experimental groups. Overall, intake progressively increased in the first days of the trial, stabilising between 20 and 30 days, with mean values between 7 and 9 kg DM/animal/d. No significant differences were found between the Control, Anavrin, and Silvafeed groups, indicating that supplementation with additives did not negatively affect the animals' intake.

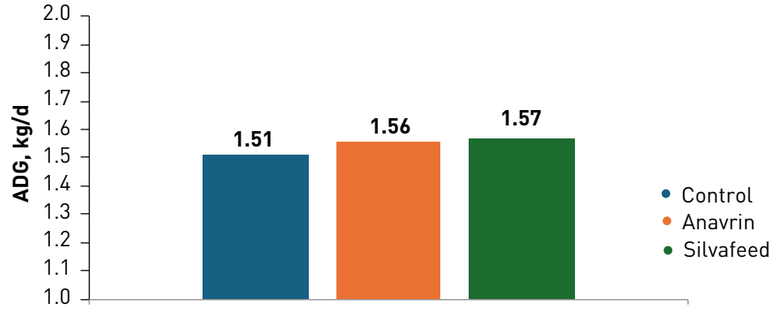
All groups showed a regular growth pattern, consistent with the physiological stages of growth, with a slight increase in intake in the final stages, corresponding to the increase in live weight and energy requirements.

Figure 3 - Individual dry matter intake in the Control, Anavrin, and Silvafeed groups



The results for average daily gains are shown in Figure 4. As can be seen from the figure, no statistically significant differences were observed between treatments ($P > 0.05$), indicating that supplementation with additives did not negatively affect growth performance.

Figure 4 - Average daily gain (ADG) in the Control, Anavrin, and Silvafeed groups



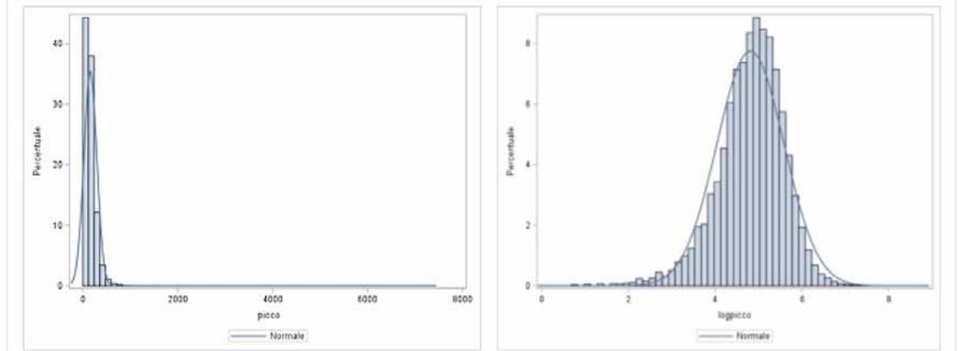
Data are reported as mean ± SEM. Treatment effect P=0.97, relief effect P=0.10, treatment x relief interaction effect P=0.31.

4.2 MEASUREMENTS OF METHANE EMISSIONS

The analysis of methane emissions revealed a non-normal distribution. For this reason, the data were expressed both on the original scale (ppm) and on a logarithmic scale (Figure 5). However, since the biological interpretation remains unchanged, the results are presented in ppm to ensure immediate and intuitive reading of the values. Furthermore, as reported in the materials and methods section, the data are reported as: means of all CH_4 values, means of all identified peaks, means of respiration peaks and eructation peaks, and number of peaks.

The interpretation of the results follows the method proposed by Laven & Yang (2025), according to which the evaluation of effects should not rigidly depend on exceeding conventional significance thresholds (e.g., P<0.05), but should focus on compatibility intervals, which describe the range of effects consistent with the data. This approach highlights both the biological plausibility of the effect and any residual uncertainty, overcoming the "significant/non-significant" dichotomy. In this study, however, a technical significance threshold of P < 0.10 was also adopted, as explained in the Materials and Methods section, useful for identifying effects consistent with biological hypotheses in a complex experimental context.

Figure 5 - Logarithmic transformation of the data



As shown in Table 2, mean methane values, expressed in logarithmic form, did not differ between the groups (CON: 4.74; ANAV: 4.72; SILV: 4.59). Analysis of the number of total peaks, respiration peaks, and eructation peaks (Table 3) also revealed no significant differences between the treatments.

However, the analysis of the data expressed in ppm revealed more interesting results, as shown in Table 4. **Specifically, the Silvafeed group showed the lowest mean methane concentration** (125.81 ppm, 95% CI: 125.32–127.39), **followed by Anavrin** (145.16 ppm, 95% CI: 144.23–146.37) and the Control group (156.90 ppm, 95% CI: 156.12–158.68).

The compatibility range for **Silvafeed** is entirely below that of the Control and does not overlap with that of Anavrin, indicating a **reduction close to 20%**, technically significant according to the threshold P < 0.10 adopted. The narrow width of the ranges suggests a sufficiently precise estimate of the effect. **Anavrin** also shows a **reduction**, but of a **more modest extent (about 7-8%)**, with partial overlapping of the control intervals.

This result differs significantly from the extensive literature on the additive Anavrin and its greater efficacy in reducing methanogenesis compared to the observed data, thus highlighting the need for further investigation. The references report, in vitro and live studies conducted in both dairy and beef cattle, with evidence also in white veal calves, effects of reduction greater than 13% on daily methane production (g/d), with effects even greater than 15% when emissions are attributed to dry matter intake (Montini et al., 2024; Minutti et al., 2025; Sgoifo Rossi et al., 2022; Altshuler et al., 2023; Grossi et al., 2024).

The trend of total peaks confirms the general pattern: **Control (170.68 ppm), Anavrin (160.89 ppm), and Silvafeed (144.47 ppm)**. Here too, Silvafeed presents the lowest consistent value, with a compatibility range indicating a consistent and physiologically relevant reduction, although with residual uncertainty compatible with the typical variability of emission peaks. The effect is significant.

The respiratory component of methane is particularly informative, as it reflects the amount absorbed through the digestive tract and then eliminated through exhalation. The recorded values are: Control was 161.75 ppm (95% CI: 158.13-163.09), Anavrin was 151.91 ppm (95% CI: 149.27-153.27), and Silvafeed showed values of 131.92 ppm (95% CI: 130.86-134.18). The compatibility range for **Silvafeed** is completely separate from that of the Control and significantly lower than that of Anavrin, indicating an effect consistent with a technically significant **18-20% reduction in respiratory emissions.** Anavrin showed a smaller reduction, although consistent with moderate physiological mitigation.

The peaks attributed to eructation did not show consistent differences between groups. The ranges are wide and overlapping, indicating that the additives' effect primarily affects rumen fermentation and systemic (respiratory) methane, rather than the mechanical dynamics of eructation.

Based on the compatibility ranges and the technical significance adopted: Silvafeed shows the most marked mitigating effect, with reductions of nearly 20% in total and respiratory emissions, supported by compatibility ranges that do not overlap with the Control. Anavrin shows a more moderate effect, but is consistent with a physiologically plausible reduction in enteric emissions. The lack of full significance (P < 0.05) for some parameters do not contradict the effect; as pointed out by Laven & Yang (2025), this simply reflects the magnitude of the statistical uncertainty, not the lack of a biological effect.

Table 2 - Methane Emission Data Expressed in Logarithmic Format (Mean and Confidence Interval, 95% CI).

VARIABLE	TREATMENT			SEM[1]	P-VALUE[2]		
	CONTROL	ANAVRIN	SILVAFEED		T	R	T×R
log CH_4 (RAW DATA)	4.74 (4.74-4.76)	4.72 (4.71-4.73)	4.59 (4.59-4.61)	0.002	0.323	<.0001	<.0001
log CH_4 (ALL PEAKS)	4.85 (4.82-4.86)	4.84 (4.82-4.85)	4.74 (4.73-4.76)	0.005	0.434	<.0001	<.0001
log CH_4 (ERUCTATION PEAKS)	5.69 (5.57-5.69)	5.70 (5.62-5.71)	5.71 (5.67-5.76)	0.014	0.976	<.0001	<.0001
log CH_4 (RESPIRATION PEAKS)	4.81 (4.79-4.82)	4.80 (4.78-4.81)	4.68 (4.67-4.70)	0.004	0.385	<.0001	<.0001

[1] SEM = Standard Error of the Mean

[2] T = Treatment
R = Relief
T×R = Treatment x relief interaction

Table 3 - Number of total peaks, respiration peaks and eructation peaks (Mean and Confidence Interval, 95% CI).

VARIABLE	TREATMENT			SEM[1]	P-VALUE[2]		
	CONTROL	ANAVRIN	SILVAFEED		T	R	T×R
CH_4 ALL PEAKS, n	90.42 (87.99-92.85)	89.02 (86.73-91.22)	90.70 (88.49-92.90)	0.666	0.572	0.140	0.694
CH_4 RESPIRATION PEAKS, n	86.22 (83.87-88.56)	84.78 (82.55-86.95)	85.81 (83.59-88.03)	0.655	0.676	0.326	0.901
CH_4 ERUCTATION PEAKS, n	4.88 (4.25-5.65)	4.73 (4.14-5.49)	5.33 (4.78-5.88)	0.185	0.428	0.002	0.118

[1] SEM = Standard Error of the Mean

[2] T = Treatment
R = Relief
T×R = Treatment x relief interaction

Table 4 - Effect of treatment, sampling day, and their interaction on methane emissions expressed in ppm (Mean and Confidence Interval, 95% CI).

VARIABLE	TREATMENT			SEM[1]	P-VALUE[2]		
	CONTROL	ANAVRIN	SILVAFEED		T	R	T×R
CH_4 ALL DATA, PPM	156.90 (156.12-158.68)	145.16 (144.23-146.37)	125.81 (125.32-127.39)	0.334	0.066	<.0001	<.0001
CH_4 ALL PEAKS, PPM	170.68 (166.00-171.45)	160.89 (157.58-162.57)	144.47 (142.25-148.04)	0.803	0.175	<.0001	<.0001
CH_4 RESPIRATION PEAKS, PPM	161.75 (158.13-163.09)	151.91 (149.27-153.27)	131.92 (130.86-134.18)	0.609	0.084	<.0001	<.0001
CH_4 ERUCTATION PEAKS, PPM	353.88 (310.81-359.49)	341.89 (307.06-366.20)	364.73 (325.93-407.66)	9.971	0.909	0.002	0.002

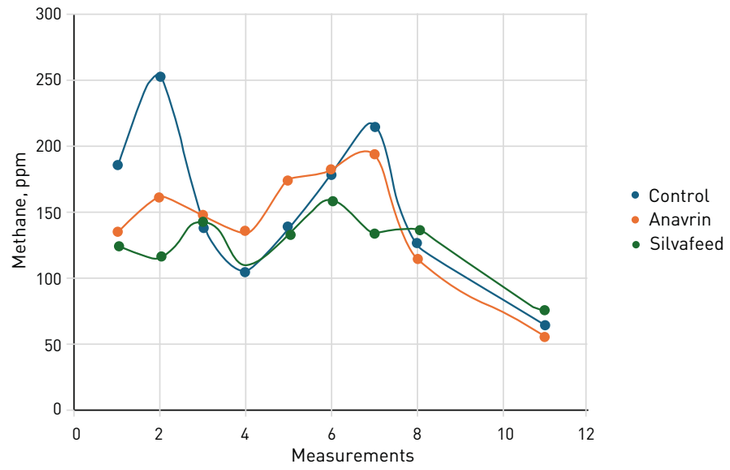
[1] SEM = Standard Error of the Mean

[2] T = Treatment
R = Relief
T×R = Treatment x relief interaction

It is also worth noting that the rations fed to the animals had a fairly high lipid content (5.73% of dry matter), a characteristic known to physiologically reduce ruminal methanogenesis. This implies that the entire experimental system was already operating under low baseline emission conditions. Therefore, observing further reductions of 15–20% (Silvafeed) or 7–8% (Anavrin) must be considered particularly significant, as they were obtained in a context where the margin for improvement was intrinsically limited. This result confirms that both supplements demonstrated a biologically interesting effect, with a differentiated impact consistent with their respective action mechanisms.

The trend in methane emissions is shown in Figure 6. The Control group showed higher average values than the Anavrin and Silvafeed treatments, especially in the initial measurements, with a maximum peak around 250 ppm, followed by a progressive decrease in subsequent measurements. The Silvafeed group, however, showed consistently lower values than the Control group for almost the entire period considered, while Anavrin exhibited an intermediate trend, with slightly higher values in the central phases but aligned with Silvafeed in the final phases.

Figure 6 - Methane emission trends in the three experimental groups



4.3 CHARACTERISTICS OF UNIFEED, RESIDUES, FAECES AND APPARENT DIGESTIBILITY

The mean characteristics of the unifeed and faeces are shown in Tables 5 and 6. The aTTD results are shown in Table 7 and in Figures 7 and 8. Table 7 shows the values for the entire test period, divided into "Covariate" and "Test." During the Covariate period, no statistically significant differences were observed between the three study groups. Conversely, during the entire Test period (10 weekly measurements), starch digestibility was significantly higher in the Anavrin group (94.78%) than in the Control group (93.31%; $P < 0.05$), with 95% confidence intervals (CI): 94.35–95.22 and Control: 92.88–93.75. The difference, equal to approximately 1.5 percentage points, although modest, suggests a consistent and reproducible improvement in digestibility in the Anavrin group. No significant differences emerged in the comparison between the two additives, Anavrin and Silvafeed, regarding starch. Compared to the Control group, the results of the Silvafeed group are at an intermediate level and show technical significance ($P = 0.06$). The 95% confidence intervals for the Silvafeed group (93.72–94.58) indicate a possible positive effect of the treatment, which is more moderate than that of the Anavrin group.

Regarding NDF digestibility, no significant difference emerged, considering both the Covariate and the entire test period, given the highly overlapping confidence intervals. Similarly, no significant differences emerged for lipid and protein digestibility (data not shown in the table). No significant effect was found regarding the interaction between treatment group and study period.

Table 5 - Compositional characteristics of the unifeed and feed residues throughout the entire trial, considering the covariate and average over the entire period of additive administration (Test). Values reported as % of dry matter

PERIOD	DRY MATTER	ASH	CRUDE PROTEIN	LIPIDS	aNDF	STARCH	ADL	AIA
UNIFEED								
COVARIATE	55.20	6.20	13.40	4.50	33.70	30.20	2.40	0.60
TEST	61.03	5.98	13.44	4.84	29.97	35.64	2.32	0.37
FEED RESIDUES								
COVARIATE	66.73	5.98	15.02	-	20.74	41.32	3.46	-
TEST	65.01	5.39	13.88	-	22.10	43.94	2.11	-

Table 6 - Compositional characteristics of faeces in the three study groups during the entire trial, considering the covariate and average for the entire period of additive administration (Test). Values reported as % of dry matter

TREATMENT	DRY MATTER	ASH	CRUDE PROTEIN	LIPIDS	aNDF[1]	STARCH	ADL[2]	AIA[3]
COVARIATE								
CONTROL	19.50	13.51	24.22	7.69	47.01	7.57	8.85	1.04
ANAVRIN	19.30	14.13	24.72	8.42	45.41	7.31	8.94	1.25
SILVAFEED	20.17	13.84	22.90	8.34	47.51	7.41	8.97	1.25
TEST								
CONTROL	21.09	12.51	21.59	8.00	49.12	8.77	8.33	1.02
ANAVRIN	19.78	13.22	21.76	7.67	50.00	7.34	8.79	0.96
SILVAFEED	19.64	12.86	21.51	7.84	49.89	7.91	8.61	1.08

[1]aNDF = Neutral Detergent Fiber
[2]ADL = Acid Detergent Lignin
[3]AIA = Acid-Insoluble Ash

Table 7 - Apparent Starch and NDF Digestibility Measured During the Covariate and the Test Period

PERIOD	CONTROL (LSMean[4] ± SE[5], 95% CI[6])	ANAVRIN (LSMean ± SE, 95% CI)	SILVAFEED (LSMean ± SE, 95% CI)
STARCH DIGESTIBILITY, %			
COVARIATE	94.21 ± 0.68 (92.87 – 95.55)	94.33 ± 0.68 (93.00 – 95.68)	94.20 ± 0.68 (92.87 – 95.55)
TEST*	93.31 ± 0.19 (92.88 – 93.75)[a]	94.78 ± 0.19 (94.35 – 95.22)[Bb]	94.15 ± 0.19 (93.72 – 94.58)[b]
P treatment 0.289			
P period 0.678			
P period*treatment 0.404			
NDF DIGESTIBILITY, %			
COVARIATE	67.82 ± 3.62 (60.61 – 75.02)	68.42 ± 3.63 (61.21 – 75.63)	66.82 ± 3.62 (59.62 – 74.03)
TEST	55.52 ± 0.74 (53.87 – 57.18)	57.36 ± 0.74 (55.70 – 59.02)	56.05 ± 0.74 (54.39 – 57.70)
P treatment 0.848			
P period <0.0001			
P period*treatment 0.956			

[4]LSMean = Least Squares Mean
[5]SE = Standard Error
[6]95% CI = Confidence Interval 95% CI

* Average of weekly measurements.
[AB] $P < 0.0001$
[ab] $P < 0.100$

Figures 7 and 8 show monthly differences in starch and NDF digestibility. Monthly analysis of the starch digestibility data showed that it progressively increased from January to April in all groups. No significant differences emerged for January, which represents the covariate, or for February. In March, the digestibility found in the Anavrin group (94.62%, CI: 93.85-95.38) was significantly higher than that of the Control group (93.39%, CI: 92.63-94.15) (P = 0.029). No differences emerged compared to the Silvafeed group, which ranked intermediate. In April, however, the starch digestibility found in the Anavrin group (95.15%, CI: 94.57-95.73) was significantly higher than both the Control group (93.26%, CI: 92.86-94.86) (P < 0.0001) and the Silvafeed group (94.29%, CI: 93.51-95.03) (P < 0.04), with significant differences also between Silvafeed and Control (P = 0.0162). In April, the confidence intervals did not overlap between Control and Anavrin and between Control and Silvafeed and showed only a low overlap between Anavrin and Silvafeed, favoring Anavrin.

No statistically significant differences emerged in the monthly trend (Figure 8) of NDF digestibility.

Figure 7 - Monthly starch digestibility values recorded in the three study groups

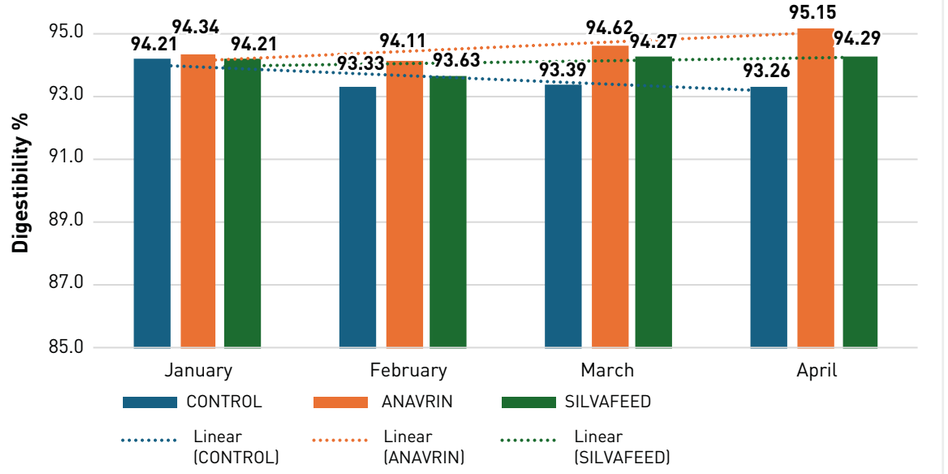
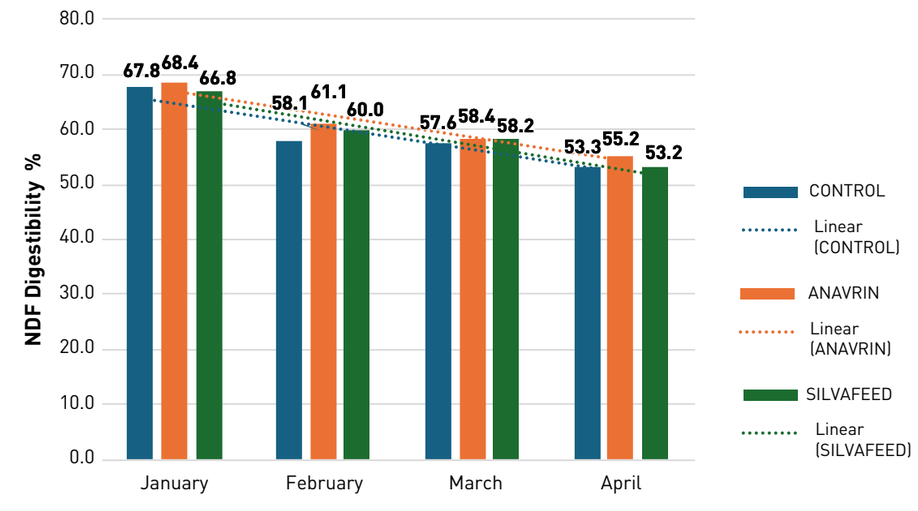


Figure 8 - Monthly NDF digestibility values recorded in the three study groups



No statistically significant differences emerged in faecal pH, either considering the mean values of the covariate and the entire test period (Table 8) or the monthly values (Figure 9).

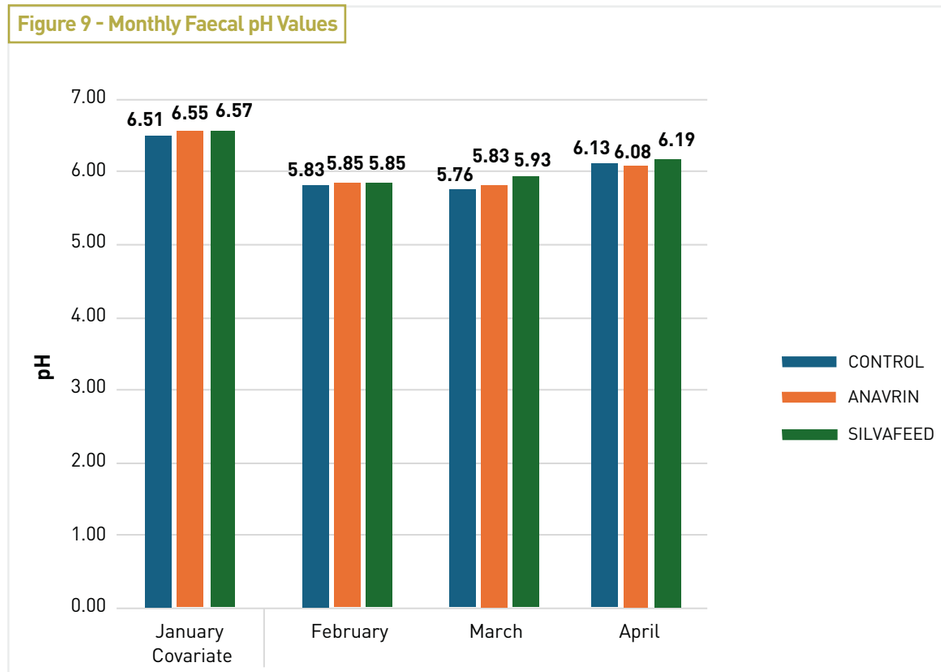
Table 8 - Mean Faecal pH Values

PERIOD	CONTROL (LSMean[1]± SE[2], 95% CI[3])	ANAVRIN (LSMean ± SE, 95% CI)	SILVAFEED (LSMean ± SE, 95% CI)
COVARIATE	6.50±0.20 (6.09-6.91)	6.55±0.20 (6.14-6.95)	6.57±0.20 (6.16-6.98)
TEST	5.95±0.04 (5.84-6.06)	5.95±0.05 (5.84-6.06)	6.04±0.05 (5.93-6.15)

P treatment 0.854
P period <0.0001
P period*treatment 0.977

[1]LSMean = Least Squares Mean
[2]SE = Standard Error
[3]95% CI = Confidence Interval 95% CI

Figure 9 - Monthly Faecal pH Values



4.4 BLOOD PARAMETERS

Regarding blood analysis results, overall, most parameters did not show significant differences between the groups, confirming that supplementation did not compromise the metabolic and hepatic status of the animals as the values fell within the physiological ranges reported for beef cattle (Kaneko et al., 2008). However, some statistically significant variations emerged (Table 9). Uric acid was higher in the supplemented groups compared to the control (P = 0.01), while a slight difference was observed for direct bilirubin (P = 0.05), with higher values in the Silvafeed group. Serum chloride also showed significant differences (P < 0.01), with higher values in the control than in the Anavrin group. Furthermore, the UIBC (latent iron binding capacity) parameter differed between the groups (P = 0.05), with lower values in the control than in the additive group. Other indices related to energy metabolism, liver function, serum proteins and mineral balance did not show significant differences.

Table 9 - Results of blood chemistry analyses of the animals included in the study

	CONTROL	ANAVRIN	SILVAFEED	*P*-VALUE
ENERGY METABOLISM				
GLUCOSE mg/dL	137	151.5	125	0.22
UREA mg/dL	24.29	24.04	26.67	0.33
URIC ACID mg/dL	1.34^{B}	1.58^{A}	1.46^{A}	0.01
TOTAL CHOLESTEROL mg/dL	143.27	154.27	143.83	0.85
LIVER FUNCTION				
TOTAL BILIRUBIN mg/dL	0.28	0.27	0.32	0.12
DIRECT BILIRUBIN mg/dL	0.05^{AB}	0.04^{B}	0.06^{A}	0.05
INDIRECT BILIRUBIN mg/dL	0.23	0.23	0.26	0.14
ASPARTATE MINOTRANSFERASE U/L	135	133	118.5	0.23
GAMMA-GLUTAMYL TRANSFERASE U/L	22.9	26.35	20.8	0.26
ALKALINE PHOSPHATASE U/L	135	150	137.5	0.62
SERUM PROTEINS				
TOTAL PROTEINS g/dL	7.2	7.03	7.02	0.56
ALBUMIN g/dL	3.54	3.68	3.58	0.26
GLOBULIN g/dL	3.66	3.35	3.44	0.32
A/G RATIO	0.99	1.11	1.06	0.32
MINERALS AND ELECTROLYTES				
SODIUM mmol/L	146	145	146	0.25
POTASSIUM mmol/L	5.8	6.05	6.4	0.73
CHLORINE mmol/L	100^{A}	97^{B}	99^{AB}	<.01
PHOSPHORUS mg/dL	8.74	8.48	8.57	0.39
CALCIUM mg/dL	9.87	9.98	9.72	0.25
MAGNESIUM mg/dL	2.27	2.37	2.25	0.3
IRON METABOLISM				
TOTAL IRON µg/dL	194.55	200.36	188.67	0.79
TIBC[1] µg/dL	481.45	536.09	502.42	0.06
UIBC[2] µg/dL	286.91^{B}	337^{A}	313.75^{AB}	0.05
SATURATION %	40.41	37.26	37.53	0.53

[1] Total Iron Binding Capacity
[2] Unsatured Iron Binding Capacity

However, some variations deserve consideration. Uric acid was significantly higher in the supplement groups compared to the Control group (P = 0.01), suggesting a possible increase in oxidative activity or a different protein metabolism, consistent with antioxidant effects described for some plant extracts (Patra and Saxena, 2011). Direct bilirubin showed slightly higher values in the Silvafeed group (P = 0.05), indicating possible liver adaptation to the different diet, without obvious clinical implications.

An interesting aspect concerns urea: although not statistically significant (P = 0.33), it was numerically higher in the Silvafeed group compared to the Control and Anavrin groups (26.67 vs. 24.29 and 24.04 mg/dL). This observation, although within physiological limits, could reflect a different nitrogen utilisation at the rumen level, plausibly linked to the presence of secondary plant metabolites capable of modulating protein degradability. The observation appears consistent with the data for direct bilirubin, outlining a slight metabolic effect specific to this additive. Finally, the differences found for chloride and UIBC indicate a modulation of electrolyte balance and iron metabolism, while remaining within physiological values. This evidence is consistent with the hypothesis that additives based on plant extracts can modulate rumen metabolism, helping to reduce enteric methane production through effects on the microbial community (Hristov et al., 2013), without compromising blood health parameters.

4.5 SLAUGHTER RESULTS

Results regarding animal performance and carcass characteristics, colour, and pH are reported in Table 10. The treatments did not significantly affect initial and slaughter body weight nor carcass weight and hot carcass yield (P > 0.05). Similarly, no significant differences were found between the experimental groups for the parameters of pH and carcass colour. However, a physiological decrease in pH was observed in the 24 hours following slaughter. Overall, the results suggest that the prolonged administration of the additives Anavrin and Silvafeed did not negatively influence either production performance or meat quality characteristics. Despite the inclusion of the additives for the entire duration of the trial, the animals reached optimal slaughter weights and maintained high quality standards. In all cases, the P values > 0.05, confirm the absence of statistically significant differences between the treatments.

Table 10 - Effect of treatment on growth performance, carcass characteristics, colour, pH, and temperature

VARIABLE	TREATMENTS			SEM[1]	*P*-VALUE[2]
	CONTROL	ANAVRIN	SILVAFEED		
INITIAL BODY WEIGHT, KG	408.3	409.6	410.7	11.1	0.997
SLAUGHTER WEIGHT, KG	589.8	605.9	604.6	8.05	0.684
HOT CARCASS WEIGHT, KG	380.0	394.1	393.3	5.49	0.522
HOT CARCASS YIELD, %	64.40	65.07	65.04	0.22	0.418
***RECTUS ABDOMINIS* MUSCLE COLOUR**					
***L**[3]*	28.12	28.33	29.74	0.456	0.291
***a**[4]*	11.21	11.24	9.99	0.348	0.246
***b**[5]*	7.83	7.13	6.88	0.276	0.367
SUBCUTANEOUS FAT COLOUR					
L*	62.19	64.62	63.15	0.610	0.273
a*	1.50	1.42	1.40	0.134	0.950
b*	7.57	5.95	6.37	0.481	0.380
***LONGISSIMUS THORACIS* MUSCLE COLOUR**					
L*	37.66	39.44	37.96	0.463	0.248
a*	17.76	17.18	17.45	0.342	0.798
b*	14.39	13.68	14.02	0.335	0.704
INTRAMUSCULAR FAT COLOUR					
L*	77.93	77.98	79.35	0.403	0.265
a*	1.85	1.83	1.99	0.166	0.916
b*	9.11	8.64	8.84	0.225	0.702
LONGISSIMUS LUMBORUM MUSCLE					
pH 45 min	6.12	6.23	6.27	0.030	0.168
pH 24 h	5.56	5.50	5.55	0.021	0.569
T°C 45 min	36.52	34.95	36.16	0.324	0.126
T°C 24 h	5.15^{A}	4.09^{B}	5.20^{A}	0.127	<.0001

[1] SEM = Standard error of the mean
[2] P-value = Treatment effect
[3] Lightness
[4] Red-green component
[5] Yellow-blue component
[AB] Within a row, values marked with the same letter uppercase are equal (P < 0.05)

4.6 SLAUGHTER DATA: ON-SITE ASSESSMENT OF PH AND PRESENCE OF LESIONS ON THE RUMEN WALL

Ruminal pH and visual assessment data are shown in Table 11. No rumens with signs of ruminitis were detected, which was therefore omitted from the table. It is interesting to note that the majority of rumens, regardless of treatment group, showed signs of infestation, even severe, with *Paramphistomum* (71% of rumens analysed).

Despite significant numerical differences in the incidence of hyperkeratotic lesions (50% in the Silvafeed group vs. 33.33 and 36.36 in the Anavrin and Control groups), the data did not reach statistical significance. Furthermore, in the Control group, animals with stellate lesions had an average of more than one lesion per animal (specifically, 2 and 8 lesions), while in both the Silvafeed and Anavrin groups, an average of 1 lesion per animal was found.

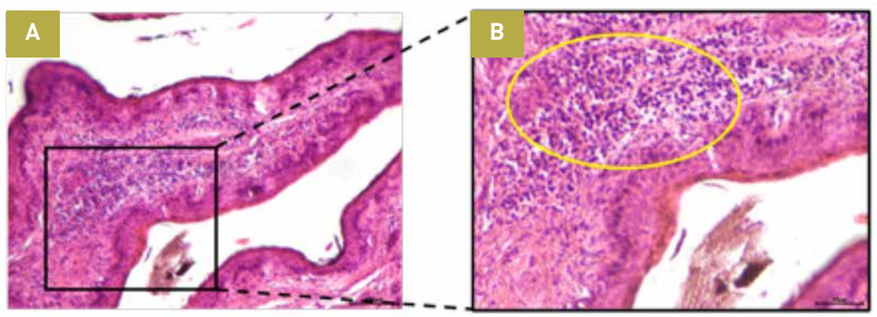
Table 11 - Rumen Parameters: pH and visual assessment

	CONTROL	ANAVRIN	SILVAFEED
N OF RUMEN EVALUATED	11	12	12
pH	6.63	6.59	6.58
HYPERKERATOSIS, % N	36.36 (4)	33.33 (4)	50.00 (6)
STAR SCARS, % N	18.18 (2)	8.33 (1)	25.00 (3)

4.7 SLAUGHTER DATA: HISTOLOGICAL AND MORPHOLOGICAL EVALUATION OF THE RUMEN WALL

From an observation of rumen morphology, the presence of lymphocytic infiltrates in the *lamina propria* is evident in samples from animals in the Control group, indicating a non-acute inflammatory process (Figure 10). However, this infiltrate was not observed with the same frequency in the Anavrin and Silvafeed treatment groups.

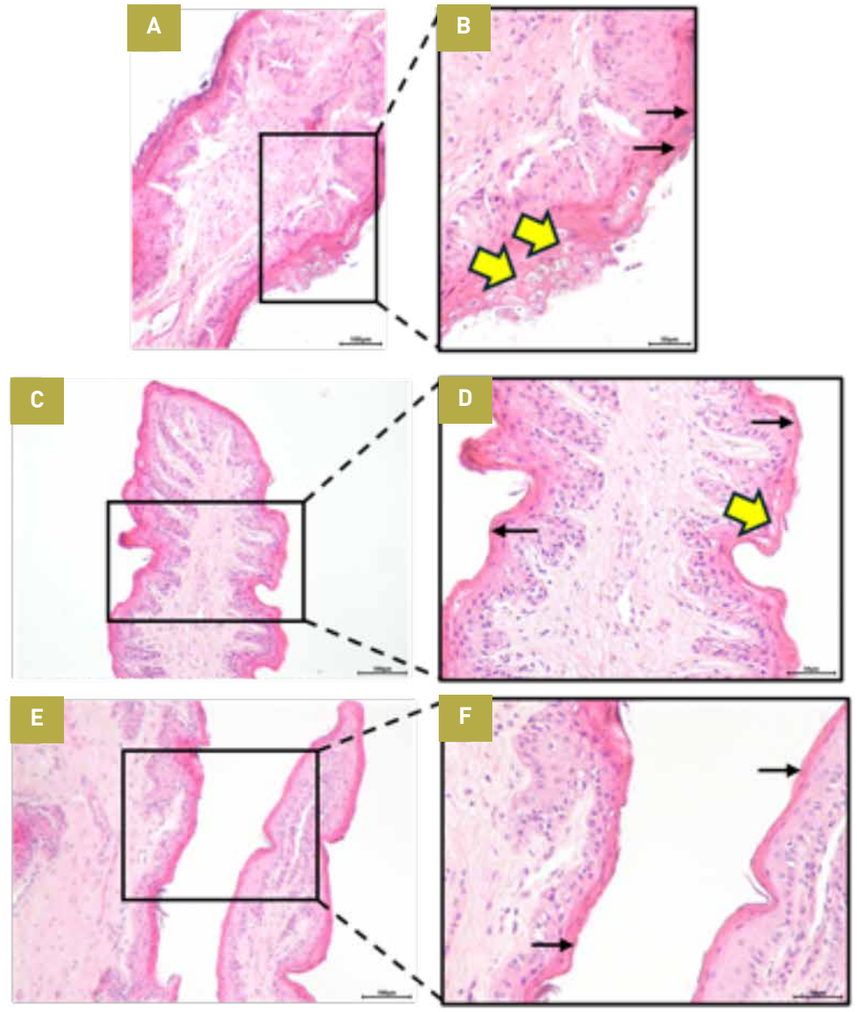
Figure 10 - Representative images of the ruminal papillae of animals in the Control group. A, 100X, B, 200X.



Yellow circle, lymphocytic infiltrate.

In all samples, keratinization appears altered due to the presence of nucleated cells in the cornified layer (Figure 11, black arrow). In particular, in the Control group samples, the presence of large, "balloon-shaped" cells, described in the literature as parakeratotic (Ragionieri et al., 2016), is evident. These cells have large spaces around their nuclei and weakly stained cellular material only at the periphery (Figure 11, yellow arrow).

Figure 11 - Representative images of the rumen papillae of animals from the Control (A and B), Anavrin (C and D), and Silvafeed (E and F) groups.



A, C, and D, 100X; B, D, and F, 200X. Black arrows, stratum corneum nuclei; yellow arrows, hypertrophic cells.

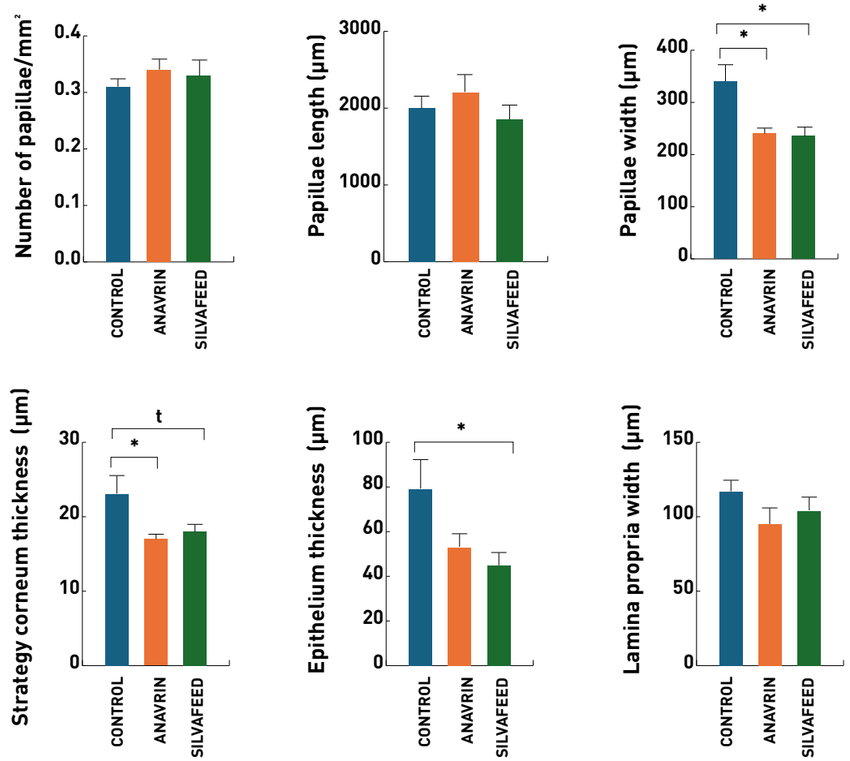
It has been described that an increased amount of organic matter available for fermentation in the rumen can lead to hyperplasia of metabolically active cells (Ragionieri et al., 2016). As a result, during the cornification process, the stratum granulosum disappears or is reduced to a very limited number of cells, and the cells of the stratum spinosum transform directly into swollen, ovoid, and still nucleated keratinocytes, constituting a thickened stratum corneum. According to Marcato (Marcato, 2000), this morphology is typical of parakeratosis and could be promoted by vacuolation of epithelial cells due to non-physiological osmolarity caused by high concentrate intake (Hinders and Owen, 1965; Nocek et al., 1984).

Table 12 - Summary of section observations: lymphocytic infiltrate, keratinization, and presence of hypertrophic cells. NO, absent; + few; ++ some; +++ many; ++++ very many

TREATMENT	SAMPLE	LYMPHOCYTE INFILTRATE	KERATIZATION	HYPERTROPHIC CELLS
CONTROL	L24-25	+++	NO	NO
	L24-26	NO	NO	+
	L24-27	NO	NO	+++
	L24-28	++	NO	
	L24-29			
	L24-30	+	NO	++
	L24-31	NO	NO	+
	L24-32	NO	NO	
	L24-33	NO	+	
	L24-34	NO	NO	+
	L24-35	+	+	+
ANAVRIN	L24-1	NO	++++	
	L24-2	NO	++	NO
	L24-3	NO	NO	+
	L24-4	+	+	+
	L24-5	NO	++	+
	L24-6	NO	+++	NO
	L24-7	NO	+	NO
	L24-8	NO	++	NO
	L24-9	NO	NO	NO
	L24-10	NO	+	NO
	L24-11	NO	+++	NO
	L24-12	NO	NO	+
SILVAFEED	L24-13	NO	NO	+
	L24-14	NO	NO	+
	L24-15	NO	+	NO
	L24-16	+	NO	NO
	L24-17	NO	NO	NO
	L24-18	NO	+	NO
	L24-19	NO	NO	NO
	L24-20	NO	+	NO
	L24-21	NO	NO	NO
	L24-22	+	+	NO
	L24-23	NO	NO	NO
	L24-24	+++	NO	NO

Histometric analyses confirm the qualitative findings. In fact, the Control group animals have a statistically significantly larger papillae width than those fed Anavrin or Silvafeed. The greater papillae width in the Control group is due to a greater thickness of the epithelium, particularly the stratum corneum (Figure 12).

Figure 12 - Histometric analysis; values are expressed as mean ± standard error of the mean



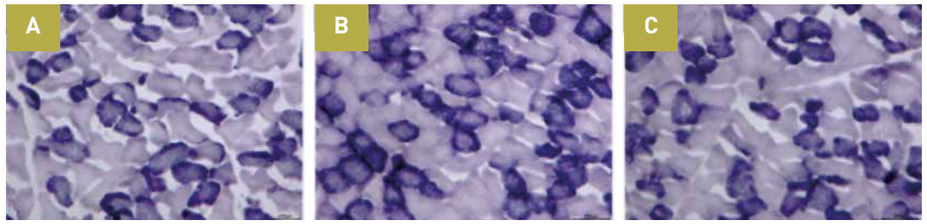
*t, trend (P < 0.07), * P < 0.05*

Considering the overall morphological and histometric analyses of the rumen mucosa, significant structural alterations associated with the basal diet were noted, characterised by incomplete keratinization and the presence of nucleated cells in the stratum corneum, indicative of parakeratosis. A further morphological finding was the presence of lymphocytic infiltrates in the lamina propria in samples from animals fed the basal diet, indicating a chronic or non-acute inflammatory process affecting the rumen mucosa. This finding suggests a persistent local immune response, likely related to structural and functional alterations of the epithelium. These changes are associated with increased availability of fermentable substrate in the rumen, which leads to hyperplasia of metabolically active cells and the disappearance or reduction of the stratum granulosum, consistent with literature reports. Animals fed Anavrin or Silvafeed showed morphological characteristics closer to those expected for a healthier rumen, suggesting a protective effect of these supplements against the keratinization process. The results suggest that the basal diet predisposes to the development of ruminal parakeratosis, while supplementation with Anavrin or Silvafeed may help preserve the integrity and functionality of the ruminal epithelium, promoting better overall digestive health.

4.8 HISTOMETRIC EVALUATIONS OF MUSCLE SAMPLES

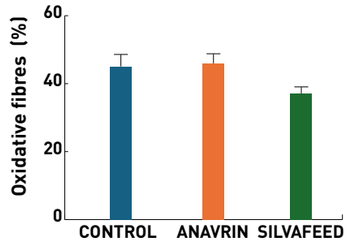
Histometric analysis of the muscle revealed no difference in the percentage of red/oxidative fibres between the experimental groups, indicating that the diet had no effect on muscle fibre metabolism (Figures 13 and 14).

Figura 13 - Representative images of the Longissimus thoracis muscle with succinate dehydrogenase (SDH) staining of the animals



Control (A), Anavrin (B), and Silvafeed (C), 100X. Blue fibres = oxidative/red.

Figure 14 - Quantification of oxidative/red fibres expressed as a percentage



Values are expressed as mean ± standard error of the mean.

5 CONCLUSIONS

This experimental study was designed and conducted using a rigorously hypothesis-driven approach with the aim of evaluating in an explicit and falsifiable way the effectiveness of plant-based feed additives in mitigating enteric methane emissions in beef cattle, as well as to assess the potential occurrence of undesirable side effects affecting production performance, carcass quality and animal welfare.

In light of the results obtained, the primary hypothesis (H1), according to which the inclusion of Silvafeed and Anavrin in the diet can reduce enteric methane emissions by modulating rumen fermentation processes, can be accepted, albeit with a differentiated effect between the two additives. Specifically, Silvafeed showed a more marked and consistent mitigating effect, with reductions of approximately 20% in total and respiratory emissions, supported by compatibility ranges that did not overlap with those of the Control group. Anavrin showed a more modest reduction, in the order of 7–8%, but it is biologically plausible and consistent with the hypothesized mechanisms of action. This result differs significantly from the extensive literature on the additive Anavrin and its greater efficacy in reducing methanogenesis compared to the observed data, thus highlighting the need for further investigation. The lack of effects on the frequency of emission peaks confirms that the action of the additives is exerted on the intensity of methanogenesis and rumen fermentation processes, rather than on the mechanical dynamics of eructation.

Regarding the secondary hypothesis (H2), intentionally formulated as a hypothesis of possible negative interference of additives on production performance, slaughter yields, carcass quality and the main biological indicators of animal welfare, the results support its rejection. The absence of significant differences in weight gain, feed intake, slaughter yield and meat quality parameters, combined with the stability of key blood chemistry indicators within physiological ranges, indicates that the reduction in emissions was not achieved at the expense of production performance, animals' metabolic status and ultimately their welfare.

Further findings of interest emerged from ruminal assessments, both macroscopic and histological, which suggest a possible protective effect of the additives on the integrity of the rumen mucosa. Animals fed Anavrin or Silvafeed showed a morphological profile closer to physiological status than the Control group; in particular, there were fewer signs of lymphocytic infiltration and impaired keratinization. Whilst not the primary objective of the study, these findings strengthen the biological interpretation of the results and suggest that modulation of ruminal fermentations can occur without compromising, and indeed potentially improving, the functional status of the rumen and the animal's health.

Overall, the confirmation of the primary hypothesis (H1) together with the rejection of the secondary hypothesis (H2) represents biologically relevant and methodologically sound evidence of the effectiveness of the studied additives in contributing to the mitigation of enteric methane emissions in beef cattle, without introducing measurable adverse effects on production, quality or animal welfare. This result is particularly significant considering that the trial was conducted in a food system already characterised by low baseline emissions, a condition that makes the observed reductions even more significant from a physiological and applicative standpoint.

Ultimately, the data obtained supports the targeted use of additives based on plant extracts as a potentially effective and sustainable tool for reducing enteric methane emissions, in compliance with the principles of experimental robustness, hierarchy of hypotheses and prudent interpretation of results, as outlined in the theoretical framework adopted in the study.

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