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FEED ADITIVES TO OPTIMIZE THE DIGESTION AND REDUCE ENVIRONMENTAL IMPACT IN LIVESTOCK

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GRIT is ... GREAT

List of abbreviations

3-NOP	3-nitrooxypropanol
ADF	Acid Detergent Fiber
ADL	Acid Detergent Lignin
AOAC	Association of Official Analytical Chemists
A:P	Acetate to Propionate ratio
BCFA	Branched-Chain Volatile Fatty Acids
BES	2-bromoethanesulfonate
C100	Cinnamon (100%)
C50-O50	Cinnamon 50% + Oregano 50%
CH_4	Methane
CH_4:VFA	Methane to Volatile Fatty Acids ratio
CP	Crude Protein
CTL	Control
DADS	Diallyl Disulfide
DM	Dry Matter
DMI	Dry Matter Intake
E100	Eugenol (100%)
E12-P12-G75	Eugenol 12.5% + Polyphenol 12.5% + Garlic 75%
E12-P75-G12	Eugenol 12.5% + Polyphenol 75% + Garlic 12.5%
E33-P33-G33	Eugenol 33% + Polyphenol 33% + Garlic 33%
E50-G50	Eugenol 50% + Garlic 50%
E50-P50	Eugenol 50% + Polyphenol 50%
E75-P12-G12	Eugenol 75% + Polyphenol 12.5% + Garlic 12.5%
EE	Ether Extract
EO	Essential Oil
FID	Flame Ionization Detector
G100	Garlic (100%)
G12-C12-O75	Garlic 12.5% + Cinnamon 12.5% + Oregano 75%
G12-C75-O12	Garlic 12.5% + Cinnamon 75% + Oregano 12.5%

G33-C33-O33	Garlic 33% + Cinnamon 33% + Oregano 33%
G50-C50	Garlic 50% + Cinnamon 50%
G50-O50	Garlic 50% + Oregano 50%
G75-C12-O12	Garlic 75% + Cinnamon 12.5% + Oregano 12.5%
GC	Gas Chromatography
GP	Gas Production
H3PO4	Phosphoric Acid
LSD	Least Significant Difference
NDF	Neutral Detergent Fiber
NH_3-N	Ammonia Nitrogen
O100	Oregano (100%)
OM	Organic Matter
P100	Polyphenol 6 (100%)
P50-G50	Polyphenol 50% + Garlic 50%
VFA	Volatile Fatty Acids

Abstract

Rumen fermentation is a major source of methane (CH_4), a potent greenhouse gas. This study aimed to evaluate the effects of various natural additives including garlic extract, polyphenols, seaweed, and essential oils on CH_4 mitigation and overall rumen fermentation. Four in vitro experiments were carried out using short- and long-term batch culture incubations to assess dose–response effects, and potential synergistic effects of combing additives, respectively. For each incubation, four different rumen inocula obtained from cattle were used, diluted with buffer (pH 6.8) at a 1:4 ratio. After 24 hours of incubation in all experiments and after 5 days in Experiments 2 and 4, pH, ammonia-N (NH_3-N), and volatile fatty acids (VFAs) were measured, moreover gas production (GP) and CH4 concentration in the gas were measured at 3, 10 and 24h in each incubation. ANOVA was performed for all parameters, considering the additive dose as a fixed effect and the animal as a random effect. Orthogonal contrasts were applied in the dose–response experiments to identify linear and/or quadratic effects.

In Experiment 1, ten additives were tested in a 24-hour batch culture system: garlic extract, eugenol, MIX containing 10% garlic, two green seaweeds, and five polyphenols (1, 2, 6, 7, and 10) with different gallic acid concentrations. Results indicated that dietary supplementation with increasing doses of Polyphenols (up to 4 mg/g DM) did not significantly reduce CH_4 emissions but were effective in lowering NH_3-N. Garlic extract supplementation (up to 12 mg/g DM) demonstrated a strong potential, reducing CH_4 emissions by up to 83% and NH_3-N levels by up to 24%, without significantly affecting total VFA concentrations. Notably, it increased propionate production by up to 68% while decreasing acetate levels by up to 32%. Eugenol supplementation showed no significant effects at the doses tested (up to 8 mg/g DM). Green seaweed supplementation did not influence CH_4 or VFA, but resulted in a moderate increase in NH_3-N when supplemented up to 200 mg/g DM.

In Experiment 2, the most promising polyphenol from Experiment 1, along with garlic extract and eugenol, were evaluated over five days consecutive batch culture incubations. Results indicated that supplementation with garlic alone (G100, 12 mg/g DM) reduced CH_4 by 78% and NH_3-N by 16%, without affecting pH or total VFA, while increasing propionate and butyrate and reducing acetate. Eugenol alone (E100) or polyphenol alone (P100) had minimal effects on CH_4 and altered VFA profiles unfavorably. Binary blends with 50% garlic (G50-E50, G50-P50) maintained strong CH4 reduction (–73%), while E50-P50 behaved like eugenol. An antagonistic effect was observed in G50-P50 as this combination led to a decrease in total VFA concentration. Ternary mixtures confirmed a garlic-dose dependency: G75-E12-P12 showed the best CH_4 reduction (–75%) and favorable VFA shifts towards propionate production, while blends with low proportions of garlic had minor effects. Long term effect of these additives and blends showed a maintained CH4 reduction bygarlic (-69%) while preserving fermentation activity, increasing GP, VFAs, and shifting fermentation toward butyrate. Eugenol's antimethanogenic effect improved over time, reducing CH_4 by 60%, but it impaired overall fermentation by decreasing VFA production. In contrast, polyphenol showed no clear effect, even after prolonged incubation. Binary and ternary blends with garlic retained antimethanogenic effects without compromising fermentation.

In Experiment 3, another set of ten additives was assessed in a 24-hour batch culture, including two garlic extracts differing in diallyl disulfide concentration (up to 12mg/gDM), cinnamon and oregano (up to 200mg/gDM), a higher dose of the MIX (up to 88mg/gDM), polyphenol6 (up to 12mg/gDM from Experiment 1, and three new polyphenols (12, 13 and 14 up to 1.2mg/gDM) with distinct gallic acid concentrations. Results confirmed the findings from Experiment 1 as Garlic extract 1 significantly reduced CH_4 (–56%), moreover a second garlic extract 2 also showed CH_4 reduction (–24%) but

impaired rumen fermentation when used at the highest dose. The MIX (70% Polyphenol 6, 20% Eugenol, 10% Garlic1) reduced CH_4 production by up to 47% with minimal impact on rumen fermentation. Supplementation with most Polyphenols (12, 13 and 14) had no effects on CH4 production and only tended to increase GP, while polyphenol 6 was able to quadratically decrease CH4 production (–21%). Cinnamon supplementation (100–200 mg/g DM) significantly inhibited CH_4 emissions by up to 98% but also GP and VFA production suggesting an excessive antimicrobial activity when used at high doses. On the contrary, no effects were noted when cinnamon was used at low doses (< 50 mg/g DM). Supplementation with oregano essential oilsreduced GP (-32%) and CH_4 (-45%) with minimal impact on VFAs when supplemented at low doses (50 mg/g DM). Higher doses (100–200 mg/g DM) nearly suppressed CH_4 (–96%) but severely impaired fermentation in terms of GP and VFA.

In Experiment 4, five-day consecutive batch culture incubation was conducted to investigate the combined effects of garlic, cinnamon, and oregano. Results indicated that garlic alone (G100) confirmed the strongest antimethanogenic short-term effects, reducing CH_4 by 78% while maintaining overall fermentation and shifting VFA from acetate to propionate and butyrate. Garlic-based blends maintained this shift and preserving high CH_4 inhibition (>70%) and moderate fermentation. Cinnamon alone (C100) and oregano alone (O100) reduced CH_4 modestly with impaired fermentation. Long-term incubations showed that Garlic (G100) was able to maintain its antimethanogenic effect reducing CH_4 (–70%) and NH_3-N (–16%) while maintaining rumen fermentation activity, however increased propionate production tended to decrease over time. Moreover, these effects were also detected in binary garlic-based blends (G50–C50, G50–O50) preserved fermentation while reducing CH_4 by 76% and 47% respectively. On the contrary, cinnamon alone (C100) and oregano alone (O100) strongly suppressed CH_4 (–90%) and NH_3-N (up to –40%) but severely impaired fermentation (up to –66% GP and –81% VFA)and shifted VFA from propionate towards acetate or butyrate. The ternary blend G75–C12–O12 exerted a strong CH_4 abatement (–86%) while maintaining overall fermentation suggesting it as the optimal long-term combination.

Overall, these findings suggest that garlic is a potent short and long-term anti-methanogenic additive able to maintain rumen fermentation in vitro even when used at high doses. However, its use could represent a challenge when used in vivo given its potential issues on palatability or odor transfer to animal products such as milk. To prevent this issue, this study demonstrated that a similar long-term decrease in CH4 production could be achieved using low doses of garlic combined with Eugenol and polyphenols (G33-E33-P33) or with cinnamon and oregano (G75-C12-O12). These results should be further investigated in vivo in order to assess the applicability of these mitigation strategies.

Key words: Additives, Essenctial oils, Garlic, Rumen fermentation.

Resumen: Uso de aditivos nutricionales para optimizar la digestión y el impacto ambiental en ganadería

La fermentación ruminal es una fuente importante de metano (CH_4), un potente gas de efecto invernadero. Este estudio evaluó los efectos de aditivos naturales incluyendo extracto de ajo, polifenoles, algas marinas y aceites esenciales sobre la mitigación de CH_4 y la fermentación ruminal. Se realizaron cuatro experimentos *in vitro* con incubaciones en cultivos no renovados de corta y larga duración, para evaluar relaciones dosis-respuesta y posibles efectos sinérgicos entre combinaciones de aditivos. Cada incubación utilizó cuatro inóculos ruminales de bovinos, diluidos 1:4 con tampón (pH 6,8). Se midieron la producción de gas (GP), la concentración de CH_4 (a las 3, 10 y 24 h), el pH, el nitrógeno amoniacal (NH_3-N) y los ácidos grasos volátiles (AGV) a las 24 h en los experimentos de corta duración, y al quinto día en los experimentos de larga duración, respectivamente. Los datos fueron analizados mediante un ANOVA considerando la dosis del aditivo como efecto fijo y el animal como efecto aleatorio. Además, se aplicaron contrastes ortogonales para identificar efectos lineales y cuadráticos. En el **Experimento 1**, se evaluaron diez aditivos en un sistema de cultivo no renovado de 24 horas: extracto de ajo, eugenol, una mezcla MIX que contenía un 10 % de ajo, dos algas verdes y cinco polifenoles (1, 2, 6, 7, 10) con diferentes concentraciones de ácido gálico como compuesto activo. Los resultados indicaron que la suplementación dietética con dosis crecientes de polifenoles (hasta 4 mg/g MS) no redujo significativamente las emisiones de CH_4, pero fue eficaz para disminuir los niveles de NH_3-N. La suplementación con extracto de ajo (hasta 12 mg/g MS) demostró un gran potencial, reduciendo las emisiones de CH_4 hasta en un 83 % y los niveles de NH_3-N hasta en un 24 %, sin afectar significativamente las concentraciones totales de AGV. Cabe destacar que aumentó la producción de propionato hasta en un 68 %, mientras que disminuyó los niveles de acetato hasta en un 32 %. La suplementación con eugenol no mostró efectos significativos a las dosis evaluadas (hasta 8 mg/g MS). La suplementación con algas verdes no influyó en el CH_4 ni en los AGV, pero provocó un aumento moderado de NH_3-N cuando se utilizó hasta 200 mg/g MS.

En el **Experimento 2**, se evaluaron durante cinco días en cultivos renovados las mezclas de diferentes proporciones de los aditivos más prometedores del experimento 1 (ajo, eugenol y polifenol 6). Los resultados indicaron que la suplementación con ajo solo (G100, 12 mg/g MS) redujo el CH_4 en un 78 % y el NH_3-N en un 16 %, sin afectar el pH ni los AGV totales, mientras que aumentó la producción de propionato y butirato y redujo el acetato. La suplementación coneugenol solo (E100) o con polifenol solo (P100) tuvieron efectos mínimos sobre el CH_4 y alteraron desfavorablemente el perfil de los AGV. Las mezclas binarias con un 50 % de ajo (G50–E50, G50–P50) mantuvieron una fuerte reducción de CH_4 (–73 %), mientras que (E50–P50) se comportó de forma similar al eugenol. Se observó un efecto antagonista en (G50–P50), ya que esta combinación provocó una disminución en la concentración total de AGV. Las mezclas ternarias confirmaron una dependencia de la dosis de ajo: G75–E12–P12 mostró la mayor reducción de CH_4 (–75 %) y un cambio favorable del perfil de AGV hacia la producción de propionato, mientras que las mezclas con proporciones bajas de ajo tuvieron efectos menores. A largo plazo, los efectos del ajo y de sus combinaciones mostraron una reducción mantenida del CH_4 (–69 %), preservando la actividad fermentativa, aumentando la producción de gas, los AGV y orientando la fermentación hacia el butirato. El efecto antimetanogénico del eugenol mejoró con el tiempo (–60 % CH_4), pero afectó negativamente la fermentación al disminuir la producción de AGV. En cambio, el polifenol no mostró un efecto claro, incluso tras una incubación prolongada. Las mezclas binarias y ternarias con ajo conservaron los efectos antimetanogénicos sin comprometer la fermentación.

En el **Experimento 3**, se evaluó otro conjunto de diez aditivos en un sistema de incubación de 24 horas, incluyendo dos extractos de ajo con diferente concentración de disulfuro de dialilo (hasta 12 mg/g MS),

canela y orégano (hasta 200 mg/g MS), una dosis más alta de la mezcla (MIX, hasta 88 mg/g MS), el polifenol 6 (hasta 12 mg/g MS, ya evaluado en el Experimento 1), y tres nuevos polifenoles (12, 13 y 14, hasta 1,2 mg/g MS) con distintas concentraciones de ácido gálico. Los resultados confirmaron los hallazgos del Experimento 1: el extracto de ajo 1 redujo significativamente el CH_4 (–56 %), mientras que el extracto de ajo 2 también logró una reducción del CH_4 (–24 %), aunque inhibió ligeramente la fermentación ruminal cuando se suministró a la dosis más alta. La mezcla MIX (70 % polifenol 6, 20 % eugenol, 10 % ajo 1) redujo la producción de CH_4 hasta en un 47 % con un impacto mínimo sobre la fermentación ruminal. La suplementación con la mayoría de los polifenoles (12, 13 y 14) no tuvo efectos sobre la producción de CH_4 y solo tendió a aumentar la GP. En cambio, el polifenol 6 logró una reducción cuadrática del CH_4 (-21 %). La suplementación con canela inhibió significativamente las emisiones de CH_4 (hasta -98 %), pero también redujo la GP y la producción de AGV, lo que sugiere una actividad antimicrobiana excesiva a dosis altas. Por el contrario, no se observaron efectos cuando la canela se usó en dosis bajas (< 50 mg/g MS). La suplementación con aceite esencial de orégano redujo la GP (-32 %) y el CH_4 (-45 %) con un impacto mínimo sobre los AGV a dosis bajas (50 mg/g MS). A dosis más altas, el CH_4 fue casi completamente suprimido (-96 %), pero la fermentación se vio gravemente afectada tanto en GP como en AGV.

En el **Experimento 4**, se llevó a cabo una incubación en cultivos renovados durante cinco días consecutivos para investigar los efectos combinados del ajo, la canela y el orégano cuando fueron combinados en diferetnes proporciones. Los resultados indicaron que el ajo solo (G100, hasta 12mg/gMS)) confirmó los efectos antimetanogénicos más fuertes a corto plazo, reduciendo el CH_4 en un 78 % mientras mantenía la fermentación general y desplazaba el perfil de AGV desde el acetato hacia el propionato y el butirato. Las mezclas a base de ajo mantuvieron este cambio, conservando una alta inhibición de CH_4 (>70 %) y una fermentación moderada. La canela sola (C100, hasta 80mg/gMS) y el orégano solo (O100, 50mg/gMS) redujeron el CH_4 de manera moderada, pero con deterioro de la fermentación. Las incubaciones a largo plazo mostraron que el ajo (G100) pudo mantener su efecto antimetanogénico, reduciendo el CH_4 en un 70 % y el NH_3-N en un 16 %, sin afectar la actividad fermentativa ruminal. Sin embargo, el aumento en la producción de propionato tendió a disminuir con el tiempo. Estos efectos también se observaron en las mezclas binarias con ajo (G50–C50 y G50–O50), las cuales conservaron la fermentación y redujeron el CH_4 en un 76 % y un 47 %, respectivamente.Por el contrario, la canela sola (C100) y el orégano solo (O100) suprimieron fuertemente el CH_4 (-90 %) y el NH_3-N (hasta -40 %), pero perjudicaron gravemente la fermentación (hasta -66 % en GP y -81 % en AGV), además de alterar el perfil de AGV, desplazando la producción desde el propionato hacia el acetato o el butirato. La mezcla ternaria (G75–C12–O12) logró una reducción importante del CH_4 (-86 %) mientras mantenía la fermentación general, lo que la posiciona como la combinación óptima a largo plazo. En conclusión, estos hallazgos sugieren que el ajo es un aditivo antimetanogénico eficaz tanto a corto como a largo plazo, capaz de mantener la fermentación ruminal in vitro incluso a dosis elevadas. No obstante, su aplicación in vivo podría representar un desafío debido a posibles problemas de palatabilidad y/o transferencia de olor a productos animales como la leche. Para evitar este inconveniente, el estudio demostró que una reducción similar del CH_4 a largo plazo puede lograrse utilizando dosis bajas de ajo en combinación con eugenol y polifenoles (G33–E33–P33) o con canela y orégano (G75–C12–O12). Estos resultados deben ser investigados más a fondo en condiciones *in vivo* para evaluar la aplicabilidad de estas estrategias de mitigación.

Palabras clave: Aditivos, Aceites esenciales, Ajo, Fermentación ruminal.

Résumé: Utilisation d'additifs naturels pour l'optimisation de la digestion et la reduction de l'impact environnemental de l'élevage

La fermentation ruminale constitue une source majeure de méthane (CH_4), un puissant gaz à effet de serre. L'objectif de cette etude est d'évaluer les effets de divers additifs naturels notamment l'extrait d'ail, les tanins, les algues marines et les huiles essentielles sur la réduction du CH_4 et la fermentation ruminale. Quatre expériences *in vitro* ont été menées à l'aide d'incubations en lot de courte et de longue durée afin d'évaluer les effets dose réponse ainsi que les effets potentiellement synergiques des différentes combinaisons d'additifs. Pour chaque incubation, quatre inoculum de rumen de bovins ont été utilisés, dilués avec un tampon (pH 6,8) dans un rapport 1:4. Après 24 h d'incubation, au cours de toutes les expériences, et au 5^{eme} jour dans les expériences 2 et 4, le pH, l'azote ammoniacal (NH_3-N) et les acides gras volatils (AGV) ont été mesurés. La production de gaz (GP) et la concentration en CH_4 ont été évaluées à 3, 10 et 24 h. Une ANOVA a été réalisée pour tous les paramètres, avec la dose de l'additif comme effet fixe et l'animal comme effet aléatoire. Des contrastes orthogonaux ont été appliqués dans les expériences dose réponse pour identifier des effets linéaires ou quadratiques.

Au cours de l'**expérience 1**, dix additifs ont été testés en incubation de 24 h : extrait d'ail, eugénol, un MIX (10 % d'ail, 20% d'eugenol et 70% de polyphenol 6), deux algues vertes et cinq polyphénols (1, 2, 6, 7, 10) avec différentes teneurs en acide gallique. Les polyphénols (jusqu'à 4 mg/g MS) n'ont pas réduit significativement le CH_4, mais ont permis de diminuer le NH_3-N. L'extrait d'ail (jusqu'à 12 mg/g MS) a montré un fort potentiel, réduisant le CH_4 jusqu'à 83 % et le NH_3-N jusqu'à 24 %, sans affecter les AGV totaux, tout en augmentant le propionate (+68 %) et en réduisant l'acétate (–32 %). L'eugénol n'a pas eu d'effet significatif (jusqu'à 8 mg/g MS). Les algues n'ont pas influencé le CH_4 ni les AGV, mais ont augmenté modérément le NH_3-N (jusqu'à 200 mg/g MS).

Pour l'**expérience 2**, le polyphénol 6, étant le plus prometteur de l'expérience 1, ainsi que l'extrait d'ail et l'eugénol, ont été évalués au cours d'incubations en batch consécutives sur cinq jours. Les résultats ont montré que la supplémentation avec de l'ail seul (G100, 12 mg/g MS) a permis de réduire le CH_4 de 78 % et le NH_3-N de 16 %, sans affecter le pH ni la concentration totale d'AGV, tout en augmentant le propionate et le butyrate, et en réduisant l'acétate. L'eugénol seul (E100, 80mg/gMS) ou le polyphénol seul (P100, 50mg/gMS) ont eu peu d'effet sur le CH_4 en plus de modifier défavorablement le profil des AGV. Les mélanges binaires avec 50 % d'ail (G50–E50, G50–P50) ont maintenu une forte réduction du CH_4 (-73 %), tandis que (E50–P50) s'est comporté comme l'eugénol. Cependnat, un effet antagoniste a été observé pour (G50–P50) puisqu' une baisse de la concentration totale en AGV a été observé.Les mélanges ternaires ont confirmé une dépendance à la dose d'ail : G75–E12–P12 a montré la meilleure réduction de CH_4 avec (-75 %) et un profil d'AGV favorable, orienté vers une production accrue de propionate, tandis que les mélanges contenant de faibles proportions d'ail ont eu des effets moindres.Les effets à long terme de ces additifs et de leurs combinaisons ont montré que l'ail maintenait une réduction du CH_4 (-69 %), tout en préservant l'activité fermentaire, en augmentant la GP, les AGV et en orientant la fermentation vers le butyrate. L'effet antiméthanogène de l'eugénol s'est amélioré avec le temps (-60 % CH_4) tout en réduisant la fermentation globale ainsi que la production d'AGV. En revanche, le polyphénol n'a montré aucun effet clair, même après une incubation prolongée. Les mélanges binaires et ternaires à base d'ail ont conservé leurs effets antiméthanogènes sans compromettre la fermentation.

Lors de l'**expérience 3**, un autre ensemble de dix additifs a été évalué dans un système d'incubation en batch de 24 heures, incluant deux extraits d'ail différant par leur concentration en disulfure de diallyle (jusqu'à 12 mg/g MS), de la cannelle et de l'origan (jusqu'à 200 mg/g MS), une dose plus élevée du

mélange (MIX, jusqu'à 88 mg/g MS), le polyphénol 6 (jusqu'à 12 mg/g MS, issu de l'expérience 1), ainsi que trois nouveaux polyphénols (12, 13 et 14, jusqu'à 1,2 mg/g MS) avec des concentrations distinctes en acide gallique.Les résultats ont confirmé les observations de l'expérience 1 : l'extrait d'ail 1 a réduit significativement les émissions de CH_4 (-56 %), tandis que le second extrait d'ail (ail 2) a également réduit le CH_4 (-24 %), mais a compromis la fermentation ruminale lorsqu'il a été utilisé à la dose la plus élevée. MIX (70 % polyphénol 6, 20 % eugénol, 10 % ail 1) a permis une réduction de la production de CH_4 allant jusqu'à 47 %, avec un impact minimal sur la fermentation ruminale. La supplémentation avec la majorité des nouveaux polyphénols (12, 13 et 14) n'a eu aucun effet sur la production de CH_4 et a seulement eu tendance à augmenter la production de gaz. Le polyphénol 6 a permis une réduction quadratique du CH_4 (-21 %). La supplémentation en cannelle (100–200 mg/g MS) a inhibé de manière significative les émissions de CH_4 (jusqu'à –98 %), mais a également réduit la production de gaz et d'AGV, ce qui suggère une activité antimicrobienne excessive à fortes doses. En revanche, aucune modification notable n'a été observée avec l'utilisation de faibles doses de cannelle (< 50 mg/g MS). La supplémentation en huile essentielle d'origan a réduit la production de gaz (-32 %) et le CH_4 (-45 %) avec un effet minimal sur les AGV à faible dose (50 mg/g MS). À des doses plus élevées (100–200 mg/g MS), le CH_4 a été presque complètement supprimé (-6 %), mais la fermentation a été gravement altérée en termes de production de gaz et d'AGV.

Par la suite, l'**expérience 4** a étudié pendant cinq jours les effets combinés de l'ail, de la cannelle et de l'origan. L'ail seul (G100, 12mg/gMS)) a confirmé les effets antiméthanogènes vu précédemment (-78 %), tout en maintenant la fermentation et en réorientant les AGV vers le propionate et le butyrate. Les mélanges à base d'ail ont conservé cette tendance avec une forte inhibition du CH_4 (>70 %) et une fermentation modérée. La cannelle (C100, 80mg/gMS) et l'origan (O100, 50mg/gMS) seuls ont réduit modérément le CH_4 mais altéré la fermentation. À long terme, l'ail (G100) a maintenu son effet (-70 % CH_4 et -16 % NH_3-N), bien que la production de propionate tende à diminuer. Les mélanges binaires (G50–C50 et G50–O50) ont permis de maintenir la fermentation tout en réduisant le CH_4 (-76 %, -47 %). En revanche, la cannelle et l'origan seuls ont fortement réduit le CH_4 (jusqu'à -90 %) et le NH_3-N (jusqu'à -40 %), mais ont fortement inhibé la fermentation (-66 % GP et -81 % AGV). Le mélange ternaire (G75–C12–O12) a réduit le CH_4 de 86 % tout en maintenant la fermentation, représentant ainsi la combinaison optimale à long terme.

En conclusion, l'extrait d'ail est un puissant additif antiméthanogène à court et à long terme, capable de maintenir la fermentation ruminale *in vitro* même à forte dose. Toutefois, son utilisation *in vivo* pourrait être limitée par des problèmes d'appétence ou de transfert d'odeur vers les produits animaux. Cette étude montre qu'une réduction similaire du CH_4 peut être obtenue avec de faibles doses d'ail combinées à de l'eugénol et des polyphénols (G33–E33–P33) ou à la cannelle et à l'origan (G75–C12–O12). Ces stratégies devraient être explorées a posteriori *in vivo* pour évaluer leur applicabilité.

Mots-clés : Additifs, Ail, Fermentation ruminale, Huiles essentielles.

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1 INTRODUCTION

Global demand for animal products is increasing due to population growth, urbanization, and rising incomes; especially in developing regions. According to the organization for economic cooperation and developpement (OECD), global milk production is projected to increase by 177 million tonnes by 2025, with an average annual growth rate of 1.8%. By 2030, milk production is expected to exceed 1,020 million tonnes, and by 2050, meat production may reach 450 million tonnes(Gerber, et al., 2013). However, this growth presents serious sustainability challenges. Livestock supply chains including feed production, enteric fermentation, manure management, and land-use changesare estimated to contribute approximately 14.5% of total anthropogenic greenhouse gas (GHG) emissions (Gerber et al., 2013). Among the three major GHGs, carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O). CH_4 is of particular concern. Although CO_2 has a much longer atmospheric lifetime (up to 200 years), CH_4 has 84 times the warming potential of CO_2 over a 20-year period and a shorter lifespan (9 to 12.5 years), making it a priority target for near-term climate mitigation (Arndt et al., 2022; Nisbet et al., 2021; Ungerfeld et al., 2022).

Livestock, particularly ruminants, are a major source of CH_4, which accounts for around 17% of total anthropogenic GHG emissions (Knapp et al., 2014). Enteric fermentation alone is responsible for approximately 6% of global emissions and represents the dominant source within the livestock sector accounting for 40% of total livestock emissions. Within this, beef and dairy cattle are responsible for 41% and 20% of the ruminant emissions, respectively (Beauchemin et al., 2022;Gerber et al., 2013). Moreover, CH_4 represents an energy loss for the animal, estimated between 2% and 12% of gross energy intake(Beauchemin& McGinn, 2006; Kinley et al., 2020).

As a result, reducing enteric CH_4 emissions has become a central focus in efforts to improve the environmental efficiency of livestock production. Among the available mitigation strategies, nutritional interventions and manipulation of the rumen microbiota stand out as practical and immediately applicable approaches (Beauchemin et al., 2020; Martin et al., 2010). These include the use of feed additives aimed at enhancing fermentation efficiency, redirecting hydrogen flow, and suppressing methanogenesis. However, their effectiveness can vary depending on the species, diet, and production system, and there are concerns about economic feasibility, animal health, and product quality. To address these challenges, researchers are exploring a range of additives such as anti-microbials, CH_4 inhibitors, defaunating agents, and plant-derived secondary compounds (Belanche et al., 2016; Patra & Saxena, 2011). These compounds can improve feed utilization by shifting volatile fatty acid (VFA) profiles toward propionate production thus reducing hydrogen availability for methanogenesis. Furthermore, considering the inefficiency of feeding ruminants with grains that could be used for human consumption, there is a renewed interest in leveraging their natural ability to convert fibrous, non-edible biomass into high-quality proteins (Beauchemin et al., 2020). Among the most promising recent interventions is 3-nitrooxypropanol (3-NOP), a synthetic compound that inhibits the enzyme methyl-coenzyme M reductase (MCR), a key catalyst in methanogenesis, effectively lowering CH_4 emissions (Martinez-Fernandez et al., 2018). Additionally, natural compounds such as halogenated metabolites from Asparagopsis spp., a red macroalga rich in bromoform ($CHBr_3$), have demonstrated strong antimethanogenic effects by disrupting the final steps of CH_4 synthesis (Machado et al., 2016; Roque et al., 2019; Vijn et al., 2020). Beyond nutritional approaches, biotechnology-based solutions such as genetic selection for low-emission animals, archaeal phage therapy, and vaccines targeting methanogenic archaea are under investigation (Hristov et al., 2015). However, these remain in early stages and face limitations due to the complexity and adaptability of the rumen ecosystem.

Given the high cost, ethical concerns, and logistical constraints of *in vivo* experiments, *in vitro* methods; particularly gas production GP systems using batch cultures are increasingly employed. These offer a standardized, reproducible, and cost-effective approach to screen dietary treatments and evaluate their impact on rumen fermentation and CH_4 production (Yáñez-Ruiz et al., 2016).

This review aims to explore the current state of knowledge regarding CH_4 mitigation strategies in ruminants, with a particular focus on nutritional interventions and their *in vitro* evaluation

2 LITERATURE REVIEW

2.1 Rumen microbial ecosystem

In ruminants, the stomach is composed of three fore-stomachs: the reticulum, rumen, and omasum; and a glandular stomach, the abomasum. Among these, the reticulo-rumen operates as a large, anaerobic fermentation chamber where microbial digestion takes place continuously (Hofmann, 1988; Umphrey & Staples, 1992). Coordinated motility patterns within the reticulo-rumen ensure effective mixing of contents, facilitate microbial colonization of ingested feed, and support the process of rumination. These contractions also aid in the passage of digesta toward the omasum and promote gas expulsion viaeructation (Church, 1993).These rhythmic contractions are critical for maintaining the physico-chemical stability of the rumen environment and ensuring efficient nutrient utilization. In order to mimic this rumen motility, several *in vitro* systems have been developed including dual flow fermenters with constant mixing, rumen simulation technique (rusitec) with vertical mixing and batch cultures with continuous or semi-continious mixing.

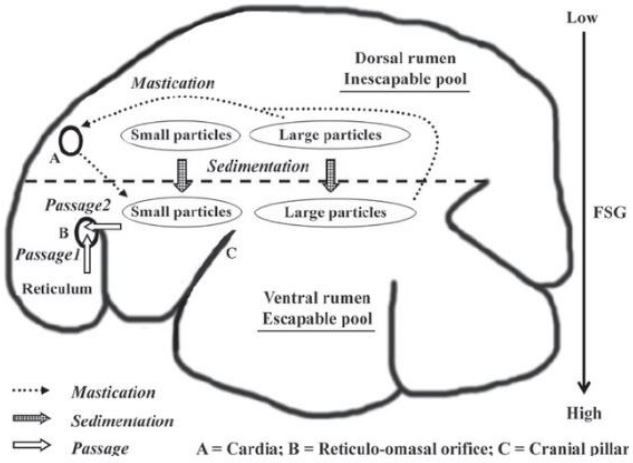


Figure 1: Distribution of conceptual pools of feed particles in the reticulo-rumen (Seo et al., 2009)

2.1.1 Physico-chemical Conditions

In cattle, the average rumen volume is approximately 150 liters, of which around 90 liters consist of digesta (Jouany, 1994). The rumen content is stratified into three distinctphases: the liquid phase, located in the ventral region; the solid phase, situated centrally; and the gaseous phase, which accumulates dorsally. Water is the principal component of rumen contents (85–90%) and is primarily found in the liquid phase, where it suspends feed particles, microorganisms, and dissolved solutes such as mineral ions. This fluid originates from various sources, including drinking water (50–100 L/day), saliva secretion (80–200 L/day), and moisture present in feed (Beede, 1993; Morgan, 2011). In contrast, the dry matter (DM) content of the rumen averages 15%, primarily located in the fibrous mass of the solid phase, which consists largely of ingested plant material(Jouany, 2006). The gaseous phase, results from both swallowed air and microbial fermentation. Composed typically bycarbon dioxide (CO_2, ~65%), methane (CH_4, 25–30%), nitrogen (N_2,~5%), hydrogen (H_2, 1–2%), and trace amounts of oxygen(Membrive, 2016).

Optimal fermentation in the rumen is maintained by strict physico-chemical parameters, including a temperature of 38–41°C, relative humidity between 85% and 90%, osmolarity ranging from 260 to 340 mOsm/kg (similar to blood), redox potential between –270 and –115 mV, and pH maintained between 5.8 and 6.8 (Church, 1993; Huang et al., 2018). These conditions are critical for sustaining microbial activity, efficient fermentation, and host nutrient absorption.

A thorough understanding of the coordinated motility and fermentation processes within the rumen is essential for the development of reliable *in vitro* fermentation models. Accurately reproducing the physico-chemical conditions of the rumen is critical in terms of pH, dilution rate, substrate availability and accumulation of fermentation products (VFA and ammonia) to maintaining representative microbial activity, and fermentation dynamics.These aspects are key to evaluate nutritional interventions under standardized conditions. Within these tightly regulated conditions, a complex and diverse microbiota operates to break down fibrous feedstuffs and support host nutrition. The following section explores the composition and functional roles of these microbial populations.

2.1.2 Rumen Microbiota

The rumen operates as a continuous anaerobic fermentation system, providing an environment that supports the growth and metabolic activity of a complex and diverse microbial community, including bacteria, protozoa, and fungi (Figure 2). Early culture-based studies identified approximately 200 cultivable bacterial species (McDonald et al., 2022). However, advances in molecular techniques, particularly high-throughput sequencing, have since revealed the presence of thousands of bacterial taxa(Janssen & Kirs, 2008), with typical population densities ranging from 10^9 to 10^{10} cells per milliliter of rumen fluid and over 1,000 bacterial taxa.Thecomposition, abundance, and functional roles of the rumen microbiota is highly dynamic and influenced by factors such as diet, host species, and ruminal environmental conditions. A summary of the major microbial groups and their general characteristics is presented in Table 1.

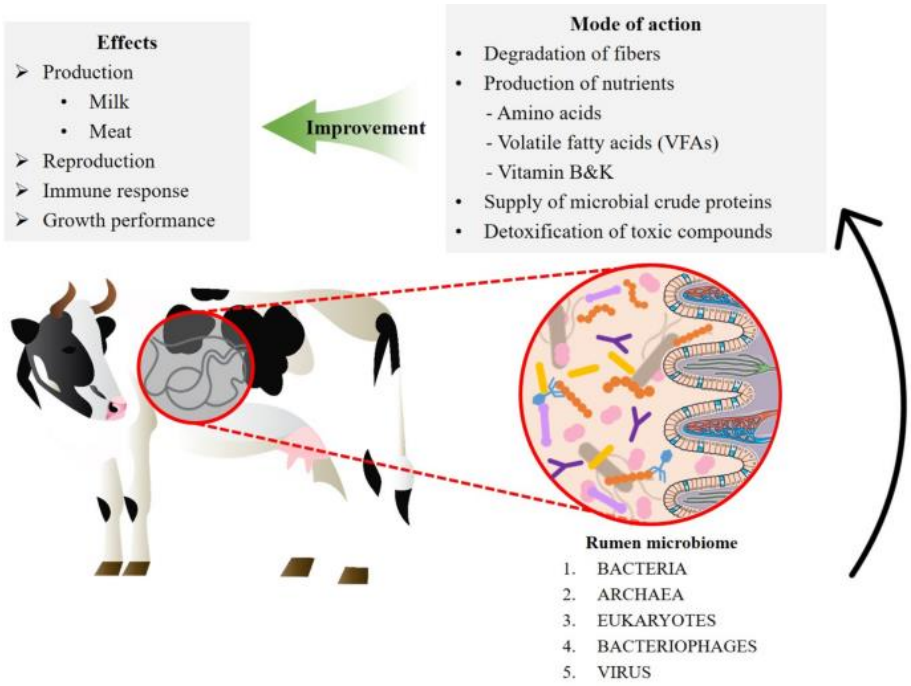


Figure 2: Schematic representation of the role of rumen microbiome (Keum et al., 2024).

Rumen bacteria are typically categorized based on their ability to degrade specific substrates, such as carbohydrates, proteins, and lipids. However, since some bacteria can degrade multiple types of substrates, certain species may belong to more than one category. These bacteria are distributed throughout both the solid and liquid phases of the rumen.

Solid-phase bacteria constitute approximately 75% of the total rumen bacterial biomass (Forsberg & Lam, 1977). This community comprises eight phyla, with *Firmicutes* being the dominant phylum, representing 42–76% of the population. This variation is mainly influenced by environmental conditions and the inherent biases of the analytical techniques used. The *Firmicutes* includes cellulolytic and hemicellulolytic bacterial species (*Butyrivibrio fibrisolvens, Ruminococcus,* which are the main fibrolytic species). This population grows best at a pH of 6.5 and above, producing mainly acetic acid (C2) and butyric acid (C4). Some fibrolytic bacteria are also amylolytic and proteolitic: this is the case of some strains of *B. fibrisolvens* and non-cellulolytic such as *Selenomonas,* a bacterium with amylolytic and proteolytic activities (Weimer, 1998; Weimer et al., 1999). The second most important phylum, in terms of proportion, is the *Bacteroidetes* representing 11 to 54% of the population. The *Prevotella* genus, which is in the majority in the rumen possesses hemicellulolytic, amylolytic and proteolytic activities (Stevenson & Weimer, 2007; Stewart et al., 1997). The third phylum, *Spirochaete*s, is in the minority in the rumen solid phase (<19%), including the *Treponemabryantii* species, which is non-cellulolytic but seems to contribute, in co-culture with a cellulolytic bacterial species such as *Fibrobacter succinogenes*, to increase the degradation of cellulose (Stanton & Canale-Parola, 1980). The *Proteobacteria* phylum is also in the minority among solid-phase bacteria (<5%), followed by four other phyla in the solid phase: *Deinococcus-Thermus* (0.1%),*Actinobacteria* (1.4%), *Verrucomicrobia* (1.7%) and *Fibrobacteres*(0.1%) (Yu et al., 2006).

Bacteria in the liquid phase of the rumen represent 25% of the total rumen bacterial biomass (Forsberg & Lam, 1977). The liquid-phase bacterial community is composed of four main phyla and are *thought* to derive mainly from the detachment of bacteria present in the solid phase (McAllister et al., 1994); these would be *Firmicutes*(11-95%),*Bacteroidetes (2-79%, Proteobacteria(1-27%) and Spirochaetes (<2%).* Therefore, it is common to observe a similarity between bacterial species in these two media. However, it has been shown that the proportion of cellulolytics is lower in the liquid phase than in the solid phase (Michalet-Doreau et al., 2001). The similarity of phyla and main genus in both rumen phases can be explained by the ubiquitous nature of these microorganisms, capable of both degrading plant walls (in the solid phase) and utilizing the products of this degradation (in the liquid phase). However, studies based on PCR-DGGE have shown that the bacterial community in the solid phase is more diverse than that in the liquid phase (Larue et al., 2005). It contains specific genera such as *Clostridium, Carnobacterium, Ruminococcus and Agrobacterium,* which are absent from the liquid phase (Cho et al., 2006). Given the important differences between the solid and the liquid associated microbiota in the rumen, several approaches have been considered to circumvent this problem when using rumen *in vitro* systems. Rumen liquid, in comparison to rumen solids, is easy to obtain and handle, as a result most *in vitro* batch cultures use rumen liquid as inoculum. More advanced *in vitro* systems such as rusitec also use rumen liquid as inoculum, but also solid digesta during the first day of incubation in order to provide solid-associated microbiota.

Other type of bacteria was identified by (Tamate et al., 1971), adhering to the ruminal epithelium. This bacterial community known as epimural-microbial community constitutes only 1% of the total rumen bacterial biomass and consequently few studies have been carried out to date to characterize its diversity (Czerkawski, 2013). The primary phyla in the rumen wall are *Firmicutes* and *Bacteroidetes,* with the genera *Butyrivibrio* (6%) and *Bacteroides* (94%) serving as the principal representatives of each

phylum, respectively (Dehority & Grubb, 1981; Mead & Jones, 1981). Most of the bacteria identified within these phyla have proteolytic activity, essential for the degradation of desquamated cells from the wall, suggesting they could stimulate sequential removal of the keratinized epithelial cells, leading to improved growth and absorptive capacity of the rumen epithelium. Therefore, tissue recycling by epimural microbiota might affect the feed efficiency of animals by recycling the host-derived nutrients and improving the cell turnover of the rumen epithelium(Cheng et al., 1979; Dinsdale et al., 1980; Na & Guan, 2022; Wallace et al., 1979). Unfortunately, no *in vitro* system has been yet able to growthis epimural microbial community.

Rumen Archaea are the only known rumen-dwelling microbes that can produce CH_4(Hook et al., 2010). They are strictly anaerobic and are referred to as methanogens. Archaea are found in the rumen in the range of $10^{6–8}$ cells/ml contributing less than 4% of the microbial community (Matthews et al., 2019). There are approximately 155 species, divided into 29 genera, 14 families, 6 orders, and 4 classes (Holmes & Smith, 2016; Janssen & Kirs, 2008; Joblin, 2005; Lee et al., 2013). Most exist freely in rumen liquid or biofilms adhering to feed particles and rumen protozoa (epi-symbiotic) or even inside of the rumen protozoa (endo-symbiotic)(Belanche et al., 2014; Janssen & Kirs, 2008; Leng, 2014). *Methanobrevibacter* is the most common rumen methanogen, accounting for 63.2% of all isolates, followed by *Methanosphaera* (9.8%) and *Methanomicrobium* (7.7%). The rest belong to the minority genera, such as *Methanimicrococcus*, *Methanosarcina* and *Methanobacterium* (Danielsson et al., 2017; A. Patra et al., 2017).

In a comprehensive analysis, Henderson et al., (2015) found that *Methanobrevibacter gottschalkii* and *Methanobrevibacterruminantium* together make up about 74% of the methanogenic population in rumen samples, regardless of the host species, geographic region, diet, or environmental conditions. So far, only 13 methanogen species from the rumen have been successfully cultured in isolation (Janssen & Kirs, 2008; King et al., 2011; Y. Liu & Whitman, 2008; Patra et al., 2017). Rumen methanogens are not easy to grow *in vitro* as they are strict anaerobes and most strains have a slow growth rate *in vitro*. As a result, the inhibitory effect of feed additives on methanogens is often studied using pure cultures rather than continuous or semi-continuous mixed cultures.

Protozoa are present in the rumen at concentrations of 10^4 to 10^6 cells/mL, encompassing more than 100 identified species, primarily belonging to the ciliate families (McDonald et al., 2022; Williams & Coleman, 1997).Most of rumen protozoa are ciliates belonging to two families. The holotrichs, ovoid organisms covered with cilia (*Isotricha* and *Dasytricha)* and oligotrichs, that includes many species that vary considerably in size, shape and appearance; as the genera *Entodinium*, *Diplodinium*, *Epidinium* and *Ophryoscolex*.Oligotrich protozoa exhibit a strong capacity to ingest suspended solid particles, such as starch granules, chloroplasts, and cellulose, using their cilia. In contrast, holotrich protozoa are more specialized in absorbing soluble sugars (Jouany., 1994). Although rumen ciliates have a lower proteolytic activity than bacteria, they are capable of breaking down insoluble proteins, demonstrating a unique function in ruminant protein metabolism. Additionally, protozoa can prey on bacteria, assimilating bacterial amino acids, peptides, and nucleic acids into their own biomass recycling microbial Nwithin the rumen, influencing the N-balance and availability for the host animal (Jouany, 1996; Ørskov & McDonald, 1979). Rumen protozoa are able to grow *in vitro*, however their growth rate is much lower than bacteria and their concentration tend to decrease over time in most *in vitro* incubation systems.

Anaerobic fungi, although less abundant than protozoa, are found in the rumen at densities ranging from 10^3 to 10^5 zoospores/mL, and play a significant role in the degradation of fibrous plant material (Akin

& Borneman, 1990; Fliegerova et al., 2015). Rumen fungi are strictly anaerobic and their lifecycle includes a motile phase (as a zoospore) and a vegetative phase (sporangium). Fungi constitute approximately 8% of the microbial biomass in the rumen. Three main genera have been identified: *Neocallimastix, Piromyces,* and *Caecomyces*. Rumen fungi develop a rhizoidal system that penetrates plant tissues and secrete a wide array of enzymes that play a significant role in the digestion of plant cell wall carbohydrates, such as cellulose and hemicelluloses (Jouany, 1994). This highlights their role in degrading recalcitrant plant materials, complementing the actions of bacteria and protozoa within the rumen ecosystem. As described for protozoa, rumen fungi have a slow growth rate which explains the low concentration often observed in most *in vitro* incubation systems.

Table 1: Composition and general characteristics of rumen microorganisms (Keum et al., 2024)

			Eukaryotes		
	Bacteria	**Archaea**	**Protozoa**	**Fungi**	**Bacteriophage**
Populations (organisms/ml)	10^{10-11}	10^{8-9}	10^{5-6}	10^{3-4}	10^{7-9}
Size (μm)	0.3-50	0.7-4	1-100	25-250	0.024-0.2
Generation interval	20 mins	25 min-6 h	8-36 h	24 h	-
Oxygen requirements	Facultative anaerobes	Strict anaerobes	Strict anaerobes	Strict anaerobes	Strict anaerobes
Predominant microorganisms in the bovine rumen	Gram negative (-) bacteria species	Methanogens (Genus *Methanobrevibacter*)	Genus *Entodinium*	Genera *Piromyces, Anaeromyces, Cyllamyces, Neocallimastix,* and *Orpinomyces*	Relative to the bacterial dominance

2.1.3 Rumen Metabolism and Efficiency

Rumen feed digestion is influenced by several factors, including diet composition, particle size, and digesta retention time which is typically reduced at high feeding levels (Huhtanen et al., 2009; Nozière et al., 2010; Nozière & Michalet-Doreau, 1996; Sauvant & Milgen, 1995). During microbial fermentation of dietary components, the rumen produces VFAs, NH_3, CO_2, and CH_4. The principal VFAs are acetate, propionate, and butyrate, which are accompanied by lesser quantities of valerate, caproate, isobutyrate, and isovalerate. Following the hydrolysis of feed polymers, monomers are metabolized through glycolysis and pyruvate to yield VFAs. Acetate and butyrate are synthesized from acetyl-CoA, while propionate is mainly produced via the succinate pathway and, to a lesser extent, the lactate pathway (Zhou et al., 2018).Volatile fatty acids represent the primary energy source for ruminants: acetate and butyrate support lipogenesis, while propionate, as the main precursor for gluconeogenesis, contributes approximately 50–60% of glucose supply. *In vitro* systems are one of the recommended methods to investigate the dietary effect on the VFA profile, however the accumulation of VFA in *in vitro* incubations represent a handicap as tend to decrease pH and inhibit microbial activity further. To overcome this problem, most *in vitro* systems imply a high dilution of the inoculum with buffer and to limit the incubation length in order to prevent excessive VFA accumulation.

Proteins are hydrolyzed in the rumen by microorganisms into peptides, amino acids, ammonia, and carbon dioxide, providing substrates for microbial protein synthesis (McAllister et al., 1994).

A portion of microbial protein is recycled in the rumen, while the remainder passes to the abomasum and small intestine, where it is digested and absorbed (Beauchemin, 2018). In protein-deficient diets, low ruminal NH_3-N concentrations (~50 mg/L) can limit microbial growth and delay carbohydrate fermentation (Bach et al., 2005). Conversely, when protein degradation exceeds microbial assimilation, excess NH_3-N is absorbed into the bloodstream, converted into urea by the liver, and mostly excreted in urine, although part is recycled via saliva(Getahun et al., 2019; Russell et al., 1992). Interestingly, some *in vitro* incubation systems mimic this salivary N recycling by incorporating urea or NH_3-N in the incubation buffer.

Lipids in ruminant diets are generally restricted to ≤50 g/kg DM, as excessive lipid intake (>100 g/kg) may alter microbial activity and fiber digestion(Noble, 1981). The primary dietary lipids are triacylglycerols rich in polyunsaturated fatty acids (PUFAs), such as linoleic (C18:2) and linolenic (C18:3) acids. In the rumen, these are hydrolyzed by microbial lipases.*Anaerovibrio lipolytica* is primarily responsible for triacylglycerol hydrolysis, while *Butyrivibrio* spp. produce esterases that act on galactolipids and phospholipids (Fay et al., 1990; C. Henderson, 1970; Privé et al., 2015)..

Dihydrogen (H_2), a byproduct of rumen fermentation, exists in both dissolved and gaseous forms, but only the dissolved fraction is available for microbial metabolism (Ungerfeld, 2015). Methanogenesis serves as a hydrogen sink to maintain ruminal redox balance, but it also represents an energy loss of up to 12% of gross energy intake (Janssen, 2010).

Nutritional inefficiencies frequently result from imbalances between protein and carbohydrate degradation rates. Excess protein degradation leads to NH_3-N accumulation and N losses, while insufficient N availability restricts microbial protein synthesis. Additionally, slow feed degradation may cause nutrient bypass and reduce utilization. In contrast, rapid fermentation, especially in high-concentrate diets, can cause lactic acid accumulation and ruminal acidosis, as only 10–20% of lactate can be metabolized to glucose (Danfær et al., 1995). Microbial protein synthesis is also impaired when ruminal pH drops below 6.0, a common occurrence during acidosis (Pathak, 2008; Russell & Wilson, 1996). Most *in vitro* systems use a high proportion of buffer in order to keeprumen pH within a phycologicalrange. Moreover, the composition of the incubation buffer can be modified to achieve the desired pH.

2.1.4 Methane Production and Mitigation Strategies

In 2020, approximately 31% of anthropogenic greenhouse gas (GHG) emissions were attributed to agro-food systems,with livestock production accounting for nearly half of this share (14.5%) (FAO, 2023; Lavagne d'Ortigue, 2022). Within the livestock sector, the largest single contributor to emissions isCH_4 from enteric fermentation in ruminants, representing 17.5% of total agro-food emissions and approximately 38% of total livestock-related emissions. Given CH4 potent global warming potential (84-87 and 28-36 times the warming potential of CO_2 over 20- and 100-years timescale, respectively) and its short atmospheric lifespan (9 to 12.5 years), reducing enteric CH4emissions is considered a key priority for achieving climate targets. Consequently, there is substantial research interest in elucidating the biological and dietary factors that drive enteric CH4 production and in identifying effective mitigation strategies to reduce emissions without compromising animal health and productivity (Garnsworthy et al., 2019; Hammond et al., 2016; Hristov et al., 2013).

2.1.5 Methane Formation in the Rumen

The composition of VFAs produced during rumen fermentation, along with CH_4 formation, plays a crucial role in determining both animal productivity and environmental impact. Rumen fermentation pathways have been intensively investigated and, to date, it is known that CO_2 and H_2 are the major precursors of CH_4 and that H_2 derives mainly from carbohydrates degradation (Figure 3). In fact, during the fermentation process, the synthesis of acetate and butyrate are accompanied by release of metabolic hydrogen, which, if allowed to accumulate in rumen fluid, has negative effects on microbial growth, and feed digestibility (Janssen, 2010). Thanks to rumen *archaea*, which manage to combine metabolic hydrogen with CO_2 in order to produceCH_4 and water as fermentation products. These microorganisms thus, regulate excess metabolic hydrogen, contributing to rumen balance while generating CH_4. Methane produced in the ruminant digestive system can be released from the animal through eructation of rumen gas (87%), exhalation after being absorbed into the bloodstream from the rumen and intestines (11%), and to a certain extent (2%) through the rectum via flatulence (Ricci, 2014).

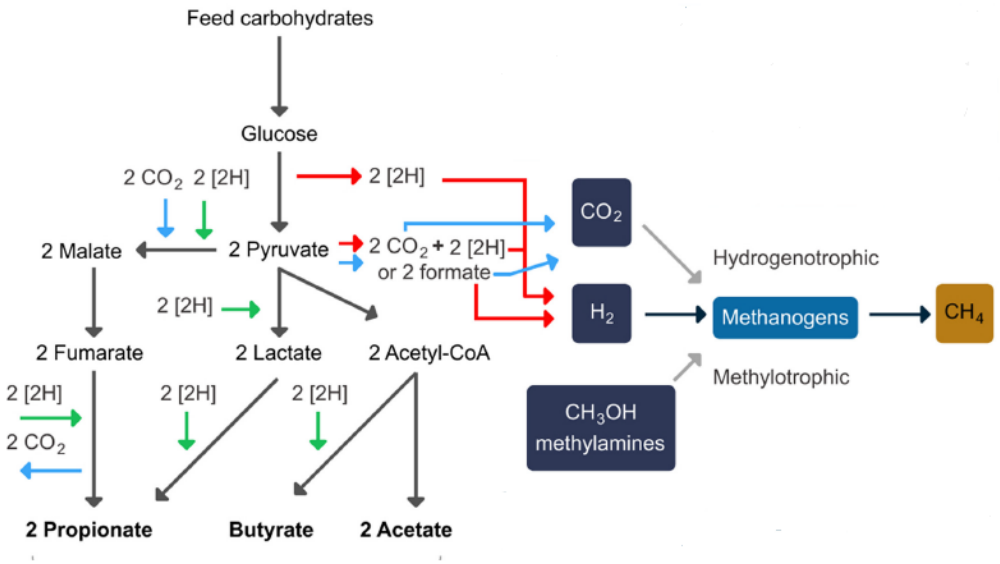


Figure 3: Illustration of carbohydrates fermentation and H_2 release in the rumen (Belanche et al., 2025)

CH_4 emissions are commonly measured using infrared spectroscopy or gas chromatography, and results are typically expressed as a percentage by volume. When evaluating CH_4 emission rates, units are generally reported in mass or volume (g or L) per unit of time. In contrast, CH_4production refers to emissions relative to feed intake, usually expressed per kg of diet consumed, whereas CH_4 yield refers to the emissions per unit of product such as milk yield or BW gain (Hammond et al., 2016).The quantity of CH_4 produced in the rumen varies depending on the pathways of glucose fermentation, as different pathways generate varying amounts of metabolic H_2, the key substrate for methanogenesis (Janssen, 2010). The amount of CH_4 emitted is also influenced by several factors, including animal species, DMI, forage type, forage-to-concentrate ratio, feed conversion efficiency, and the presence of plant secondarymetabolites (Gbenou et al., 2024; Hidayat et al., 2024; Mills et al., 2008; Palangi et al., 2022; Tseten et al., 2022).According to Liu & Whitman, (2008), rumen methanogens utilize H_2 to reduce carbon compounds to CH_4 through three principal methanogenic pathways (Figure 4):

1. Hydrogenotrophic pathway:
   •$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$
   • $4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$
2. Acetoclastic pathway:
   • $CH_3COOH \rightarrow CH_4 + CO_2$
3. Methylotrophic pathway:
   •$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$
   •$4CH_3–NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_3$
   •$2(CH_3)_2–NH + 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_3$
   • $4(CH_3)_3–N + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_3$

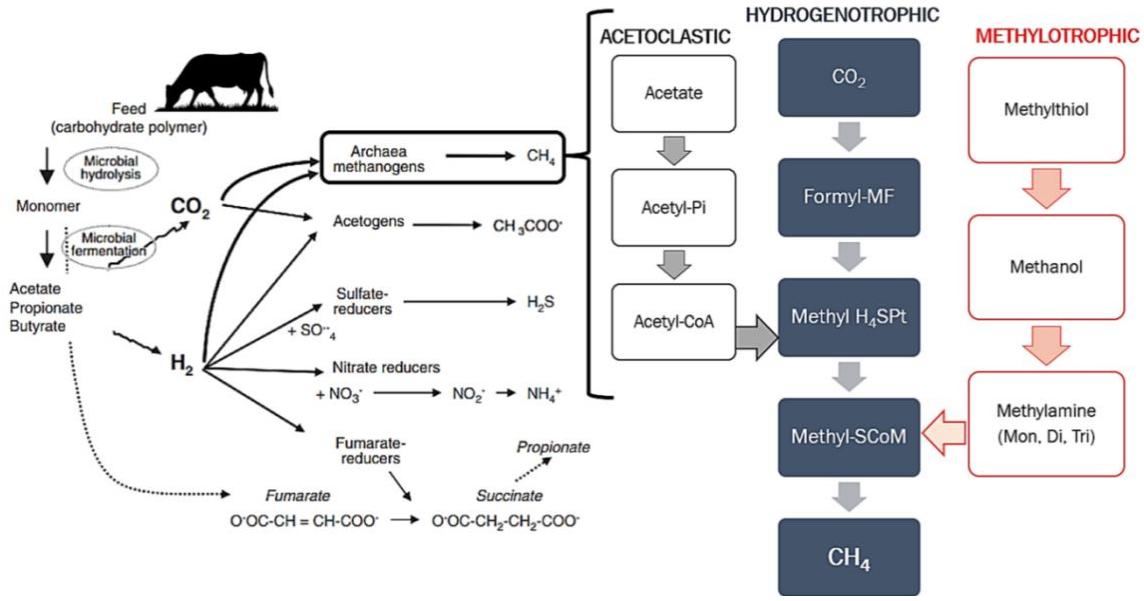


Figure 4: Methane production pathways through ruminal fermentation in cattle (Cuervo et al., 2025)

Among these, the hydrogenotrophic pathway is the most prevalent in the rumen. It involves the stepwise reduction of CO_2 using H_2 as the primary electron donor, with formate playing a secondary role (Ungerfeld, 2020). Although this pathway supports lower microbial growth rates and biomass yields, it dominates due to its favorable thermodynamics(Fenchel et al., 2012). Methanogens of the genus *Methanobrevibacter*, especially *Mbb. gottschalkii* and *Mbb. ruminantium*, have been strongly associated with elevated CH_4 emissions in steers (Wallace et al., 2015), heifers(Cunha et al., 2019), and dairycows(Danielsson et al., 2017). In contrast, *Methanosphaera*, a methylotrophic genus, has been negatively correlated with CH_4emissions (Cunha et al., 2017; Ramayo-Caldas et al., 2019). This contrast is partly due to stoichiometric differences: while the hydrogenotrophic pathway requires 1 mol of CO_2 per mol of CH_4, the methylotrophic pathway requires 4 mol of methanol to produce 3 mol of CH_4. Interestingly, methylotrophic methanogenesis appears to be more prevalent in young calves and is associated with higher NH_3-N (Friedman et al., 2017; Poulsen et al., 2013). Recent metagenomic and metatranscriptomic studies have revealed that higher CH_4 emissions are often linked to lower methanogen diversity. Cattle emitting less CH_4 typically harbor a more diverse methanogen population, encompassing all three pathways, whereas high emitters are dominated by hydrogenotrophic methanogens(Martínez-Álvaro et al., 2020). These findings highlight the role of microbial diversity and H_2 competition in determining CH_4 output (Pereira et al., 2022). All three methanogenic pathways share a key enzymatic step catalyzed by methyl-coenzyme M reductase (MCR), which is responsible for the

final reduction of methyl-coenzyme M to CH_4(Chen et al., 2020) Because of its central role, MCR has emerged as a prime target for antimethanogenic interventions.Given the central role of methanogenic pathways in ruminal fermentation, numerous strategies have been developed to mitigate CH_4 emissions without compromising productivity. These approaches are categorized and discussed below.

2.1.6 Methane Mitigation Strategies

A range of strategies has been explored to mitigate enteric CH_4 emissions in ruminants, including dietary interventions, feed additives, microbiome modulation, immunization, and genetic selection (Arndt et al., 2022). While several approaches show promising results, their practical application is often limited by economic constraints and potential risks to animal, human, or environmental health. Therefore, rigorous assessment of their effectiveness, feasibility, and safety remains essential for sustainable implementation.

2.1.6.1 Modulation of rumen fermentation to decrease H_2 production

Modulating rumen fermentation offers a promising strategy to reduce CH_4 emissions without compromising animal productivity. Strategies such as dietary manipulation, use of plant secondary compounds (e.g., tannins, saponins and essential oils), and other feed additives as described in Figure 5, target the microbial ecosystem to decrease H_2 production and ultimately rumen methanogenesis. These interventions mostly aim to enhance propionate production and modulate the fermentation kinetics to decrease rumen methanogenesis. *In vitro* fermentation systems provide a valuable platform for evaluating such strategies under controlled conditions, enabling detailed analysis of gas production, fermentation end-products, and microbial responses.

- ***Dietary manipulation***

The extent of CH_4 production in the rumen depends largely on the dominant fermentation pathways and the balance of reducing equivalents [2H] generated and consumed. Fermentation to acetate and butyrate generates molecular H_2, whereas propionate formation consumes per mole of glucose fermented as follows:

- Butyrate formation: $C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2CH_2COO^- + CO_2 + 2\ H_2 + H^+$
- Acetateformation: $C_6H_{12}O_6 + 2\ H_2O \rightarrow 2\ CH_3COO^- + 2\ CO_2 + 2\ H^+ + 4\ H_2$
- Propionate formation: $C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2COO^- + 2\ H_2O + 2\ H^+$

Generally, elevated ruminal H_2 concentrations favor propionate formation, while lower H_2 levels promote the production of acetate.Shifting fermentation patterns toward propionate reduces the net availability of H_2 and ultimately decreasing CH_4 production (Janssen, 2010).Therefore, dietary manipulation is among the most practical and cost-effective strategies for mitigating enteric CH_4 emissions in ruminants (Haque, 2018; Kebreab et al., 2010). Enhancing forage quality and optimizing the forage-to-concentrate ratio can markedly influence ruminal fermentation dynamics. High-quality forages typically contain more fermentable carbohydrates, lower levels of indigestible fiber, and reduced carbon-to-nitrogen (C/N) ratios. These characteristics favor the production of propionate, a key alternative H_2 sink that competes with methanogenesis by reducing H_2 availability (Beauchemin et al., 2009; Hills et al., 2015). One practical mean of inducing this shift is through the inclusion of concentrates in the diet. Diets containing 35–60% concentrate have been shown to lower CH_4 emissions while supporting improved animal productivity (Agle et al., 2010; Tseten et al., 2022). However, excessive inclusion of concentrate can disrupt ruminal pH homeostasis and increase the risk

of metabolic disorders such as sub acute ruminal acidosis (SARA), aconditioncharacterized by persistent, mild ruminal acidosis with negative impacts on health and performance (Abdela, 2016; Owens et al., 1998). Certain legume forages; such as Medicago sativa and Lotus corniculatusalso contribute to CH4 mitigation due to their content of condensed tannins, lower fiber fractions, and faster ruminal passage rates (Beauchemin et al., 2008). Furthermore, feed processing techniques, including chopping, pelleting, steam flaking, and alkali treatment, can alter the physical and chemical properties of feedstuffs. These modifications influence digestion kinetics and microbial accessibility to substrates, and indirectly affecting CH_4 production (Boadi et al., 2004; Knapp et al., 2014; Martin et al., 2010)

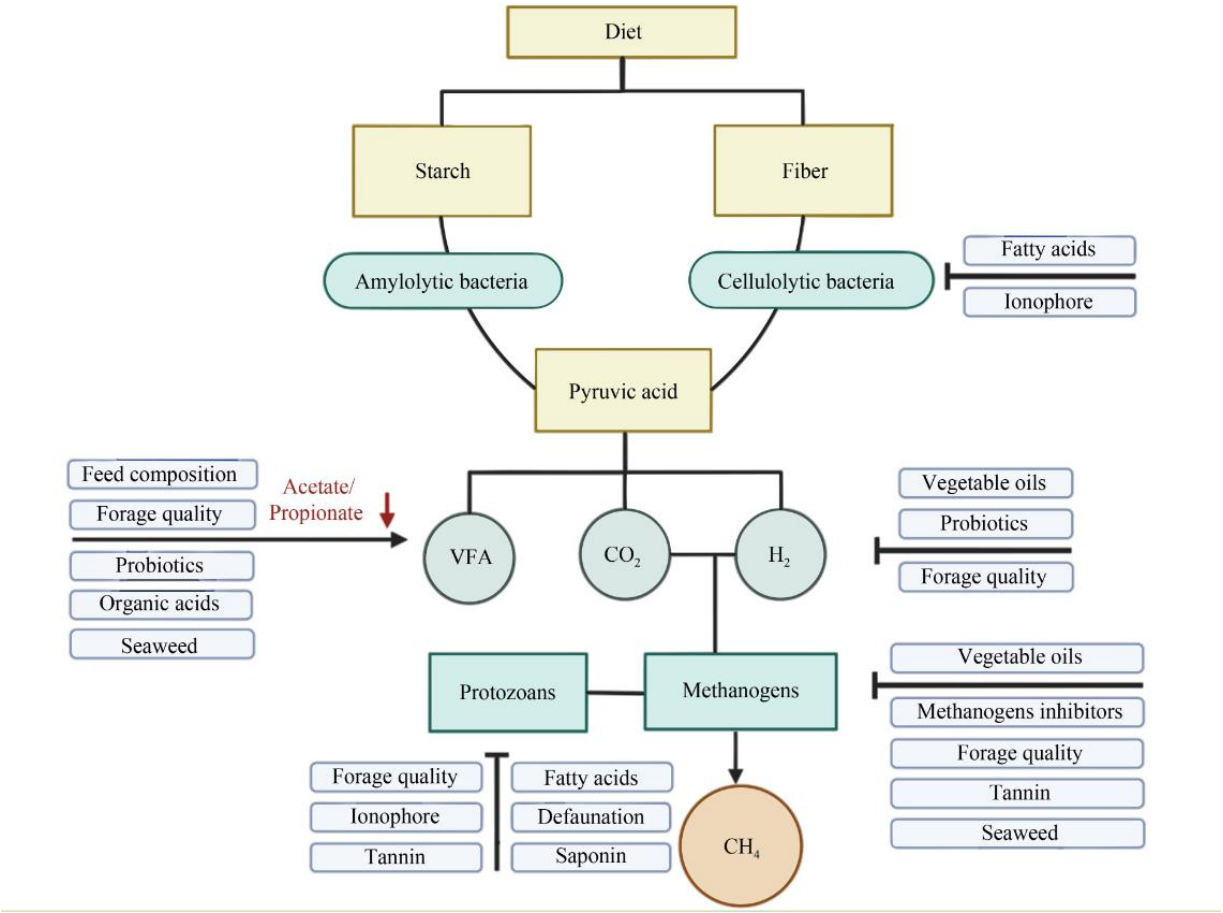


Figure 5*:* Nutritional strategies for CH_4 emission abatement in ruminants (Tseten et al., 2022)

- ***Feed additives***

In general, feed additives incorporated into ruminant diets to mitigate (CH_4 emissions act either by directly inhibiting methanogenic archaea or by modulating ruminal metabolic pathways to reduce the availability of substrates required for methanogenesis (Honan et al., 2021). This section focuses on these latter effects.

i) Saponins

Saponins are surface-active glycosides consisting of an aglycone (sapogenin) and a glycone (saccharide). Although primarily synthesized by plants, they can also be produced by certain marine organisms and specific bacterial strains (Negussie et al., 2017; Ramos-Morales et al., 2017). In ruminant nutrition, major dietary sources of saponins include *Yucca schidigera*, *Quillaja saponaria*, *Camellia*

sinensis, and *Medicago sativa*(Jayanegara et al., 2014). Saponins exert an indirect antimethanogenic effect mainly through their ability to suppress ciliate protozoa (defaunation). Structurally, they possess a hydrophilic sugar moiety and a hydrophobic steroidal or triterpenoid aglycone (Newbold et al., 2015), enabling them to interact with membrane sterols, destabilize cellular membranes, and induce cell lysis (Patra & Saxena, 2009). The efficacy of saponins in reducing CH_4 emissions varies substantially depending on their molecular structure, botanical origin, inclusion level, and the composition of the basal diet (Patra, 2012). Moreover, different types of saponins exhibit variable effects on rumen fermentation. For instance, *Y. schidigera* extracts have shown significant CH_4-inhibiting properties, whereas *Q. saponaria* extracts have produced inconsistent results (Pen et al., 2006). Most of the evidence on saponins' antimethanogenic potential comes from *in vitro* experiments, and the findings remain inconsistent across studies (Islam & Lee, 2019; Ku-Vera et al., 2020).

ii) Tannins

Tannins are another group of plant-derived compounds that influence the rumen environment and contribute to CH_4 mitigation. They are broadly classified into two types: hydrolysable tannins, which are polyesters of gallic acid and various sugars, and condensed tannins, which are polymers of flavonoids (Liu et al., 2011; Tavendale et al., 2005).

Under rumen conditions, hydrolysable tannins break down into simpler phenolic and non-phenolic compounds that may exhibit antimicrobial properties and influence fermentation dynamics. In contrast, condensed tannins primarily modulate rumen fermentation by reducing nutrient degradability and limitingCH_4 production (Nawab et al., 2020).Hydrolysable tannins generally exhibit a stronger antimethanogenic effect than condensed tannins, although their systemic absorption raises concerns about toxicity (Chen et al., 2021; McSweeney et al., 2001). Overall, tannins appear to reduce CH_4 production through both indirect and direct mechanisms: by inhibiting hydrogen-producing microbes (thereby possibly reducing fiber digestion), and by directly suppressing methanogens (Kumar et al., 2014; Tavendale et al., 2005). Supplementation with tannin-rich plants has been reported to decrease daily CH_4 emissions and reduce CH_4 per unit of energy intake by up to 24%. However, this is often associated with reduced organic matter and fiber digestibility (Tiemann et al., 2008).

Jayanegara et al. (2012) emphasized that higher dietary tannin concentrations lead to decreased CH_4 production when expressed per unit of digestible organic matter. However, the impact of tannins on CH_4 emissions is highly variable and depends on several factors, including the source and type of tannins, their molecular weight, and the rumen methanogen community(Aboagye & Beauchemin, 2019). Mechanistically, tannins exert their effects through the binding of their phenolic hydroxyl groups to protein residues via hydrogen bonding and hydrophobic interactions (Vasta et al., 2019). This tannin–protein complexation can alter protein conformation, leading to denaturation, aggregation, and changes in enzymatic activity, while also being toxic to protozoa (Kumar et al., 2014; Obreque-Slier et al., 2010). It is important to note that the reduction in CH_4 emissions induced by tannins is sometimes accompanied by a decrease in DMI and nutrient digestibility. Additionally, the efficacy of tannins is closely modulated by their concentration in forages or supplements, with effective antimethanogenic activity generally occurring at concentrations up to 20 g/kg of DMI.

iii) Flavonoids

Flavonoids are a class of secondary plant metabolites structurally related to tannins and recognized for their broad range of biological activities, including antimicrobial, anti-inflammatory, and antioxidant effects(Oskoueian et al., 2013). In the context of rumen fermentation, flavonoids are believed to reduce CH_4 emissions by modulating microbial populations and rumen fermentation pattern. One alterntive mechanism involves the cleavage of their carbon ring structures, enabling them to absorb molecular H_2, thereby decreasing the substrate available for methanogenesis (Oskoueian et al., 2013; A. Patra et al., 2017).Several studies have reported that specific flavonoids, such as naringin and quercetin, can lower CH_4 production by decreasing populations of ciliate protozoa and hydrogenotrophic methanogens like *Methanosarcina* spp., while simultaneously promoting the abundance of beneficial microbial groups such as *Megasphaeraelsdenii*, a lactate-utilizing bacterium that enhances propionate synthesis. These shifts in microbial communities are often accompanied by increased propionate concentrations, alternative H_2sink without compromising overall fermentation efficiency. Despite promising results from *in vitro* experiments, the effects of flavonoids *in vivo* remain largely underexplored. The structural diversity of flavonoids, along with variations in their bioavailability, dosage, and interaction with dietary components, contribute to the variability in their efficacy. Nevertheless, the existing evidence suggests that flavonoids possess considerable potential as natural feed additives for reducing CH_4 emissions while preserving rumen functionality.

iv) Lipids

Lipids are considered as a promising nutritional strategy for CH_4 mitigation in ruminants. Actually, when supplemented in the diet, lipids can reduce CH_4 emissions primarily by lowering the abundance of methanogenic archaea and ciliate protozoa through several mechanisms, including direct toxicity, reduction of fermentable substrate availability, and shifts in fermentation pathways (Machmüller et al., 2003; A. Patra et al., 2017). However, in some cases (more than 70g/kgDM), lipid supplementation may negatively affect fiber digestion and overall diet digestibility (Wang et al., 2023). The antimethanogenic effects of lipids are largely attributed to the action of fatty acids, particularly unsaturated ones, which can disrupt archaeal cell membranes. The integrity of these membranes is essential for maintaining chemiosmotic gradients, energy metabolism, and nutrient transport. Disruption of the membrane structure leads to ion imbalance, leakage of intracellular contents (such as potassium), and impaired enzymatic activity, ultimately resulting in methanogen cell death and reduced CH_4 synthesis. Indeed, lipid supplementation has been shown to reduce CH_4 emissions by an average of 14% when added at levels providing approximately 34 g/kg (DM) (Beauchemin et al., 2007). The effectiveness of this strategy depends on several factors, including the form, source, fatty acid profile, and degree of saturation of the lipid, as well as the composition of the basal diet(Jordan et al., 2006; Patra, 2013). Medium- and long-chain fatty acids such as lauric acid (C12:0) and alpha-linolenic acid (C18:3) have shown greater CH_4-reducing potential due to their role as H2 sinks during biohydrogenation, thereby decreasing H2 availability for methanogenesis.

Interestingly, lipid supplementation appears to be more effective in concentrate-based diets than in forage-based systems, which may limit its practical application in grazing systems (Beauchemin et al., 2022;Patra et al., 2017). Nonetheless, lipids remain attractive additives because they are widely available, generally safe for both animals and humans, and relatively easy to incorporate into total mixed rations (TMRs), especially in intensive production systems. The optimal inclusion rate of lipids in ruminant diets should consider the animal's physiological stage, the existing fat and nutrient profile of

the basal diet, and the specific characteristics of the supplemental oil source (Palmquist & Jenkins, 2017).

v) Essential oils

Essential oils (EOs**)** are volatile, aromatic compounds extracted from various parts of plants, including flowers, seeds, leaves, roots, and bark. They possess broad-spectrum antimicrobial properties and are generally recognized as safe for animal and human consumption(Tavendale et al., 2005) Their potential to reduce CH_4 emissions arises from their ability to alter ruminal microbial populations, including protozoa and methanogens, and to interfere with key fermentation pathways.The mechanism of action of EOs is believed to involve disruption of microbial cell membranes, enzyme inhibition, and interference with proton gradients, all of which can impair microbial metabolism and survival (Wallace, 2004). Notably, the antimicrobial activity of EOs does not target a single group of microbes but may involve multiple targets within bacterial cells, leading to broader shifts in microbial ecology. The extent and nature of these effects depend heavily on the EO's chemical composition, dosage, and interaction with the diet.*In vitro* studies have reported impressive reductions in CH_4 production up to 90% in some cases after EO supplementation (Busquet et al., 2005; Soliva et al., 2011). However, these promising results have not always translated into consistent outcomes *in vivo*, where microbial adaptation and dose limitations often reduce efficacy (Belanche et al., 2020; Benchaar et al., 2008; Saro et al., 2018). Moreover, the pleasant aroma of certain EOs as cinnamon, eugenol or thymol may enhance feed intake (Benchaar, 2016; Chaves et al., 2008; Yang et al., 2010) whereas strong-smelling compounds like garlic oil can reduce palatability. Another challenge is the lack of standardized regulations regarding the safe inclusion levels and toxicity of EOs in ruminant diets. As their use increases, it is essential to establish clear guidelines to ensure both efficacy and safety in practical applications. Despite these challenges, essential oils remain a promising natural strategy for mitigating enteric CH_4 emissions, especially in combination with other additives or dietary interventions.

vi) Ionophores

Ionophores are carboxylic polyether antibiotics produced by Streptomyces species, widely used in beef cattle production to improve feed efficiency, modulate rumen fermentation, and reduce CH_4 emissions(McGuffey et al., 2001). Their mechanism of action involves increasing the permeability of cell membranes in Gram-positive bacteria and protozoa to specific ions (e.g., H^+, Na^+, and K^+), thereby disrupting cellular ion gradients and inhibiting microbial growth. In the rumen, ionophores such as monensin selectively inhibit Gram-positive bacteria, including many hydrogen-producing fibrolytic and proteolytic species, as well as ciliate protozoa. This microbial shift favors Gram-negative bacteria, such as *Fibrobacter succinogenes*, and promotes fermentation pathways that yield more propionateand less acetate and butyrate (Morvan et al., 1996; Schären et al., 2017). Consequently, hydrogen availability for methanogens is reduced, leading to lower CH_4 output. Ionophores do not act directly on methanogenic archaea but exert indirect antimethanogenic effects by decreasing the production of their key substrates, such as hydrogen and formate. Studies have shown that monensin supplementation in feedlot diets improves nitrogen and energy utilization while reducing enteric CH_4 emissions (Vyas et al., 2014).However, the long-term effectiveness of ionophores remains controversial, as rumen microbes may adapt, potentially diminishing their efficacy over time. In addition, rising concerns over antimicrobial resistance, along with increasing societal and regulatory pressure to limit the use of antibiotic-based growth promoters in livestock production, have significantly reduced the acceptability of ionophores as a sustainable CH_4 mitigation strategy (Beauchemin et al., 2008).Overall, while ionophores offer a well-documented short-term reduction in CH_4 emissions and improved feed efficiency, their long-term utility is constrained by regulatory and public health considerations.

Alternative, non-antibiotic strategies are therefore being increasingly explored to ensure sustainable CH_4 mitigation in ruminant production systems.

***vii)* Rumen defaunation and vaccination**

Ciliate protozoa produce large quantities of H_2 due to the presence of hidrogenosomes. Furthermore, they form intimate associations with methanogens located on the surface and within protozoal cell. Protozoa, therefore, supply a substrate for methanogenesis while also protecting symbiotic archaea from oxygen toxicity. It is estimated that protozoa-associated methanogens contribute roughly 37% of rumen CH_4 emissions (Belanche et al., 2014). Holotrich protozoa are thought to be more efficient H_2producers than entodiniomorphids and, thus, have a greater impact on methanogenesis. A defaunated rumen results in a 10–13% drop in CH_4 production, an increase in propionate concentration, and lower levels of acetate and butyrate in the rumen content (Eugène et al., 2004; Morgavi et al., 2010; Newbold et al., 2015). Indeed, defaunation boosts bacterial population density, bacterial protein synthesis efficiency, and N flow to the duodenum, especially when the feed is low in protein relative to its energy content. Thus, if simple but permanent methods of defaunating the animals can be discovered, defaunation has the potential to be a mitigation strategy.

The development of vaccines for limiting methanogenesis is based on inducing the animal's immune system to produce antibodies in saliva, which upon entry into the rumen, should suppress the growth of methanogens (Subharat et al., 2016). Vaccination is a very appealing strategy for reducing enteric CH_4 emissions. It appears that this method would be especially beneficial for pasture-based breeding. To be effective, a vaccination must generate sufficiently high quantities of antibodies in the saliva, bind to the appropriate antigens of methanogens in the rumen fluid, and particular antigens over the whole spectrum of target methanogen species. All the *in vitro* studies showed a reduction in the amount of CH_4 released, ranging from 7 to almost 70%, depending on the type of antibodies and the immunisation protocol (Baca-González et al., 2020). When comparing studies to assess the possibilities of using vaccines against methanogens, several issues arose, and results were inconclusive (Baca-González et al., 2020; Króliczewska et al., 2023). There are few reports applying vaccines to mitigate CH_4 production from enteric fermentation in ruminants (Cook et al., 2008; Zhang et al., 2015). A vaccine against protozoan antigens has also been reported, but it failed to significantly reduce the ciliate population in Merino sheep (Leng, 2014). Therefore, the effectiveness of this strategy is complicated to evaluate, further studies are required to reach a firm conclusion on its feasibility, practicality, and long-term viability (Baca-González et al., 2020).

2.1.6.2 Alternative Hydrogen acceptors

As previously discussed, the amount of CH_4 produced in the rumen is closely linked to the availability of molecular H_2, which is itself determined by the predominant fermentation pathways. In addition to modifying fermentation patterns, alternative H_2 acceptors such as nitrate (NO_3^-) and sulfate (SO_4^{2-}) can be incorporated intoruminant diets, typically at low concentrations as electron acceptors. These compounds exhibit a higher reduction potential than CO_2 and are thermodynamically more favorable for specific rumen microbes (Kristjansson et al., 1982). As such, they serve as competitive pathways for H_2 utilization, reducing CH_4 emissions either directly by depriving methanogens of H_2, or indirectly via their intermediates, such as nitrite (Zijderveld et al., 2011).

Nitrate is commonly administered as calcium, sodium, or potassium salts and has been widely studied for its CH_4-mitigating effects in ruminants. It reduces CH_4 emissions by serving as an alternative

H2sink, thus competing with methanogens for H_2. This can occur directly, or indirectly via its intermediate, nitrite, which can also inhibit methanogenesis.Aboagye & Beauchemin, (2019) reported that dietary nitrate supplementation reduced CH_4 emissions by10-20%, without significantly affecting NDFdigestibility. However, other studies found that inclusion of nitrate at 15 g/kg DMreduced DMI by up to8%, likely due to reduced palatability and bitter taste (Benchaar & Greathead, 2011; J.-P. Jouany & Morgavi, 2007). In such cases, the reduction in CH_4 emissions may be partly attributable to lower feed intake rather than a direct inhibitory effect on methanogenesis. A major concern with nitrate supplementation is the accumulation of nitrite, its intermediate metabolite. Nitrite can impair the oxygen-carrying capacity of hemoglobin by forming methemoglobin, and has been associated with toxic and potentiallycarcinogeniceffects (Fewtrell, 2004). To mitigate this risk and enhance CH_4 reduction, nitrate utilization requires a progressive microbial adaptation process during several weeks. Moreover, nitrate utilization is often combined with other strategies such as lipid supplementation, which may help limit nitrite accumulation while synergizing antimethanogenic effects.

Sulfate (SO_4^{2-}) functions similarly to nitrate as alternative H2 sink, providing a thermodynamically favorable electron acceptor pathway for sulfate-reducing bacteriain the rumen. These microorganisms reduce sulfate to hydrogen sulfide (H_2S) in a process that consumes H2 and limits its availability for methanogenesis. From a thermodynamic standpoint, sulfate reduction is more favorable than methanogenesis, as it consumes eight electrons and offers the same H2 sink capacity per mole as nitrate (Ungerfeld & Kohn, 2006).Supplementation with sulfate salts has been shown to reduce daily CH4emissions by approximately 16**%**, and the combined use of sulfate and nitratesaltsresulted in additive effects on CH_4 mitigation. These findings highlight the potential of both compounds as effective strategies for reducing enteric CH4 production (van Zijderveld et al., 2010). However, the accumulation H_2S, a byproduct of sulfate reduction can pose respiratorydistress andimpairment of cellular respiration in ruminants. Consequently, the safe and effective use of sulfate in ruminant diets depends on factors such as inclusionlevel, diet composition, and the animal's physiological capacity to detoxify H_2S.

2.1.6.3 Inhibition of methanogens

The most effective strategies to reduce enteric CH_4 emissions are those based on the use of chemical compounds that directly inhibit methanogens (Khampa& Wanapat, 2006). These compounds must consistently lower CH_4 production without causing harm to humans, animals, or the environment, to be cost-effective and adopted by producers

- ***3-nitrooxypropanol and halogenated compounds***

3-nitroxypropanol (3-NOP) is one of the most effective dietary supplements for cattle that have been evaluated (Alvarez-Hess et al., 2019). The mechanism of action involves inhibiting the enzyme microbial CH_4 formation (Methyl-coenzyme M reductase), which is responsible for the final step in CH4 production by methanogenic archaea in the rumen (Duin et al., 2016).3-NOP is extensively metabolized into several compounds, including 3-nitrooxypropionic acid (NOPA), 3-hydroxypropionic acid (3-HPA), nitrate, nitrite, and carbon dioxide(G. Yu et al., 2021). Studies have shown that the use of 3-NOP significantly reduces enteric CH_4 emissions per day and per kilogram of DMI, with reductions ranging from 20% to 35%. In parallel, a notable increase in H_2 emissions was observed representing an energy waste as it was not used to increase animal productivity(Hristov et al., 2015). To address this, Liu et al., (2022) proposed combining 3-NOP with fumarate, which may help mitigate H_2 accumulation acting as a H_2 sink and enhance the inhibition of methanogenesis. Additionally, it has been noted that high NDF content in the

basal diet negatively affects the efficacy of 3-NOP.In addition to 3NOP, several halogenated sulfonated compounds, including 2-bromoethanesulfonate (BES), 2-chloroethanesulfonate (CES), and 3-bromopropanesulfonate (BPS), are structural analogs of methylated coenzyme M (methyl-CoM). They competitively and specifically inhibit the activity of MCR, a key enzyme in methanogenesis, thereby lowering CH_4 production at relatively low concentrations (Patra et al., 2017a; Patra, 2012). However, these compounds are carcinogenic and they cannot be used as feed additives.

- ***Seaweed***

Recently, algae have become one of the subjects of research aimed at reducing CH_4 emissions from ruminants. Particular attention is given to three main taxa of macroalgae, commonly known as seaweed, which represent a large domain of aquatic plants separated into Chlorophyta (green), Phaeophyceae (brown), and Rhodophyta (red). In general, seaweeds contain polysaccharides, proteins, peptides, lipids, phlorotannins, saponins, and alkaloids that are known to reduce CH production by suppressing archaea and protozoa. However, the mode of action responsible for the mitigation effect relies on their content in volatile halogenated compounds (bromoform CHBr3) (Machado et al., 2016). Bromoform inhibitCH_4formation by impeding the transfer of a methyl group to the enzyme MTR involved in the rumen methanogenesis. The best-studied species exhibiting CH_4 emission properties are the red seaweeds *Asparagopsistaxiformis*and*Asparagopsisarmata*due to their high content in bromoform. *In vivo* studies reported dose and diet-dependent decreases from 30 to 70% of CH_4 production by algae preparation (Stefenoni et al., 2021; Sun et al., 2023). Additionally, in some small-scale studies on cattle supplemented with algae, researchers have found a significant or numerical increase in milk yield, and feed efficiency in addition to the reduction of emission (Kinley et al., 2020; Lean et al., 2021). The Environmental Protection Agency (EPA) classified CHBr3 in Group B2 as a probable human carcinogen and toxic substance for the environment (i.e., ozone depletion). Additionally, chronic oral exposure of animals to high concentrations of $CHBr_3$ can result in liver and intestinal tumors, being this an aspect that can limit the adoption of *Asparagopsis* as a mitigation strategy.

2.1.6.4 Enhancement of productivity

- ***Genetic selection***

Genetic selection represents a very attractive CH_4 mitigation strategy because changes are cumulative and permanent. Over the last decade, researchers have demonstrated that the heritability of CH_4 formation and emission in dairy cattle was moderate, ranging from 0.11 to 0.33; however, the heritabilities of CH_4 yield in sheep were higher (0.24–0.55) (Pickering et al., 2012; Sypniewski et al., 2021). Animal selection is a very long-term process and selecting animals with low CH_4 emissions looks rather like an excellent future strategy (Króliczewska et al., 2023).
Manzanilla-Pech et al., (2021)concluded that, when compared to CH_4 production (g/d), CH_4 yield (g/kg DMI), and CH_4 intensity (g/kg energy-corrected milk), residual CH_4 has the greatest potential for inclusion in the breeding goal because it allows for selecting low CH_4-emitting animals without compromising other economically and physiologically important traits (e.g. low productivity or small rumen). However, it requires multidisciplinary investigation and a large number of animals with CH_4 records, where reliable biomarkers are needed to estimate CH_4 production on all types of farms. Moreover, while long-term strategies are undoubtedly important, there is an urgent imperative to initiate immediate reductions in $CH_4$and other greenhouse gas emissions from livestock production.

2.1.6.5 Methods of quantifying enteric CH4production

Accurate measurements of CH_4 emissions from ruminants under diverse conditions are essential for developing effective CH_4mitigation strategies. Over the past three decades, various technologies have been employed worldwide to measure enteric CH_4 emissions from ruminants. These methods vary in application, cost, accuracy, and precision, yet all direct approaches are based on measuring CH_4concentrations in the air. Historically, CH_4 emission assessments aimed to quantify energy losses as CH_4 within the energy balance and estimate heat production through respiratory exchange measurements (Reynolds et al., 2014). However, this approach required the use of expensive chambers. In recent years most studies focus on the direct measurement of CH_4 outputs using both *in vivo* and *in vitro* approaches.

2.1.7 *In vivo* Methods

2.1.7.1 Respiration chambers (RC)

Respiration chambersoperate by collecting the animal's exhaled breath and flatulence through an integrated system of inlet and outlet pipes, aflowmeterand gas analyser allowing the measurement of CH_4concentration and its daily variation (Broucek, 2014). The principle for determining CH_4emissions in RC is to measure the difference in CH_4concentration of air flowing in and out of the chamber, multiplied by the airflow through the system, corrected to a standard temperature, pressure and humidity (Mathot et al., 2016; Pinares-Patiño et al., 2011; Waghorn et al., 2014). The advantage of RC is their ability to measure CH_4 kinetics throughout the day with high precision. However, they cannot be used on pasture or farms. Their operation demands a high level of expertise, including control of gas recovery and ventilation rates making them more suitable for research in experimental stations (Huhtanen et al., 2019). The main weakness of RC lies in the animal restrictions, which do not reflect a normal husbandry environment and may affect DMI(Della Rosa et al., 2021).

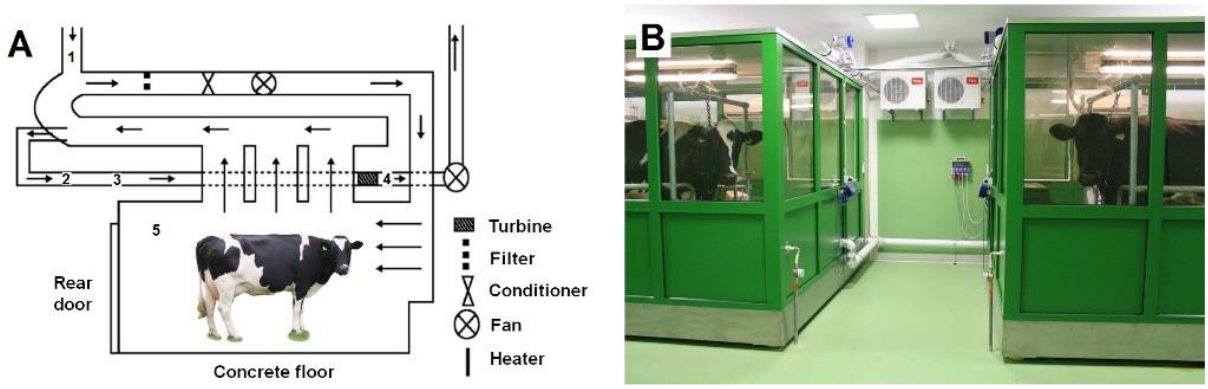


Figure 6: (A) Schematic diagram of the open-circuit respiration chamber showing air fluxes(B) (Grainger et al., 2007)

2.1.7.2 Sulfur hexafluoride tracer technique

Sulfur hexafluoride (SF_6) tracer technique was adapted to the estimation of CH_4 emissions from ruminants by (Johnson et al., 1994). The basic idea behind the method is that CH_4 emission can be measured if the emission rate of a tracer gas from the rumen is known. SF_6 was selected because it is physiologically inert, non-toxic, it has a very low detection limit, and is easy to analyze(Johnson et al., 1994; Zimmerman, 1993). The technique involves the controlled release of SF6 from a permeation tube

placed in the rumen, with continuous breath sampling through a line connected from the nostrils to a pre-evacuated canister. CH_4 production is then estimated by multiplying the SF_6 release rate by the CH_4:SF_6 ratio in the collected sample, after adjusting for background concentrations of both gases (Hammond et al., 2016). Although the SF_6 tracer technique remains a practical method for measuring CH_4emissions from grazing animals, it has certain limitations that can affect accuracy. Factors such as variability in permeation tube release rates, adjustments for background gas concentrations, and discrepancies between SF6-based estimates and chamber measurements contribute to significant within- and between-animal variation.

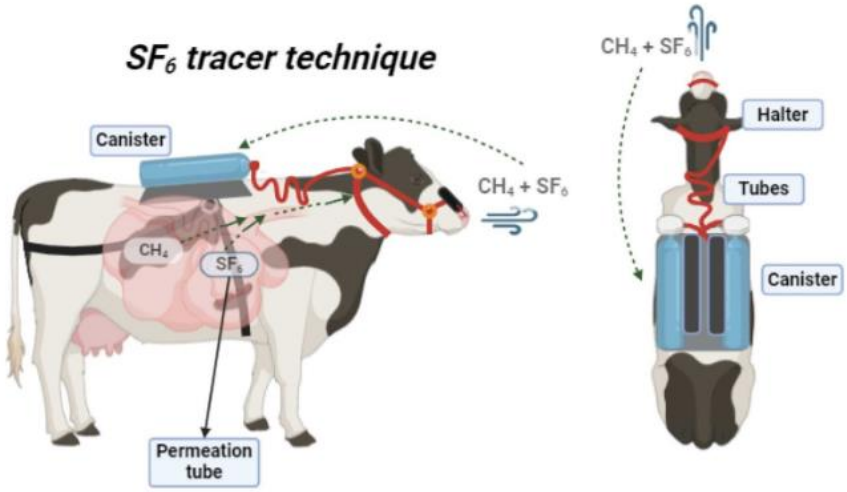


Figure 7: SF_6 Tracer technique for Measuring Enteric (CH_4) Emissions in Dairy Cows"

2.1.7.3 The GreenFeed system

The GreenFeedsystemis a short term measurement device that measures CH_4 and CO_2 from individual cattle, measuring airflow, gas concentration during each animal's visit to the unit (Zimmerman & Zimmerman, 2012). The system measures gas emission using a combination of an extractor fan and sensors to create a measured airflow past the animal's head. The device detects gas emissions and samples the released air (Huhtanen et al., 2015). CH_4 emission measurements with a GreenFeed unit are generally conducted at brief intervals (3–7minutes), multiple times daily, across several days, weeks, or months, contingent upon each animal's voluntary attendance at the GreenFeed unit. The concentration of CH_4, CO_2, and O_2 in the sample is quantified using non-dispersive infrared analysis. This system's is less stressful for animals than RC and is applicable in a variety of environments, including grazing conditions (Hristov et al., 2015). The main limitation is the cost and the time needed for the animals to get adapted to the system.

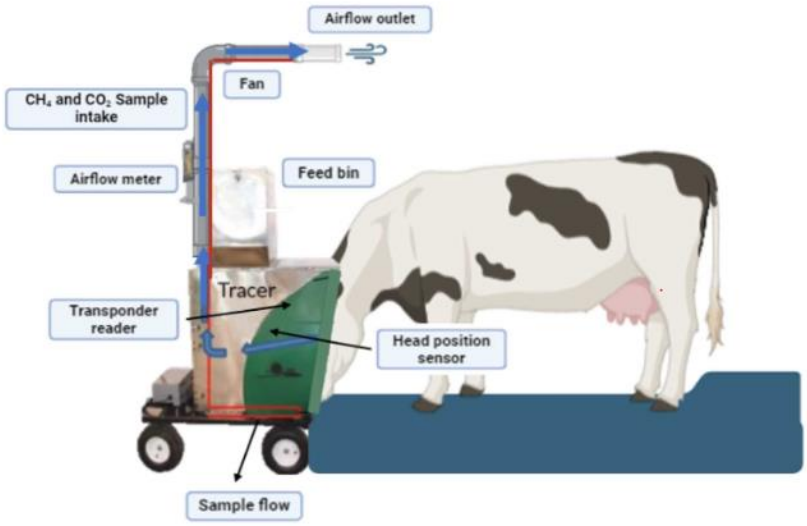


Figure 8: The GreenFeed system for Measuring Enteric (CH_4) and Carbon Dioxide (CO_2) Emissions in Dairy Cows"

2.1.8 *In vitro* Methods

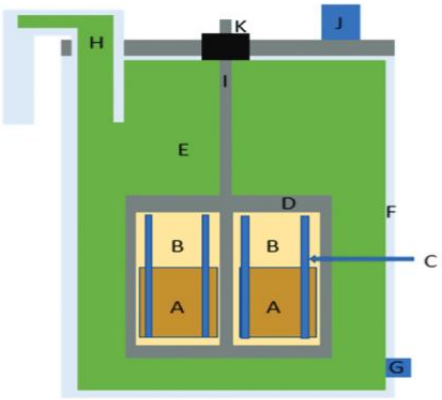
Given the limitations of animal experiments, due to costs, time, ethical concerns, and standardization constraints, there is growing interest in non-animal research methods, like *in vitro* rumen fermentation experiments. The *in vitro* batch systems developed for gas production GP and adapted for measuring CH_4 show promise for evaluating various additives or comparing different dietary treatments regarding their effects on rumen fermentability (in terms of GP) and CH_4 production (Yáñez-Ruiz et al., 2016)

2.1.8.1 Batch culture

Tilley & Terry, (1963)developed the batch culture incubation methodology for the *in vitro* fermentation of feed ingredients, further the technique was updated by Goering & Van Soest, (1970). These methodologies require the collection of ruminal fluid, diluting the fluid with buffer, and incubating it in closed bottles with the substrate of interest (Yáñez-Ruiz et al., 2016). Following incubation, the contents are filtered and analysed to determine the digestion that occurred. While simple, batch culture has a wide variety of analyses that it can be used for, including GP, fermentation end products, nutrient degradation, and microbial communities. As batch culture is a fully closed system, GP measurements are simple and can measure through changes in pressure in the bottle and concentrations of different gasses therein (Theodorou et al., 1994). The current batch culture methodology allows for evaluation of the quality of fermentation and extent of nutrient degradation throughout incubation. This allows for the evaluation of fermentation profiles and end-products (VFAs, NH_3-N, pH, and microbial ecology) as well as the degradation of nutrients. A distinct advantage of batch culture is the ability to test a large number of treatments at one time. The main limitation of this technique is that some rumen microbes are not able to grow *in vitro*, as well as the high ratio incubation volume / substrate and the short incubation time (e.g. 24h) which are needed to prevent an excessive accumulation of fermentation products. These limitations often make difficult to extrapolate the doses used *in vitro* to subsequent *in vivo* studies.

2.1.8.2 Continuous culture fermentation

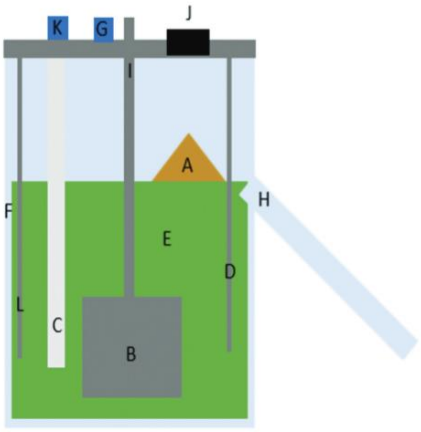
Continuous culture fermentation (CC) was originally described by (Hobson, 1965) and involves the maintenance of an *in vitro* culture of ruminal fluid, over a longer period of time. CC shares with all *in vitro* experiments the advantage of being low experimental cost, the ability to test treatments with sufficient statistical power in a short amount of time, and the possibility of exploring effects that cannot be studied *in vivo*. But, CC has one distinct advantage over the other types of *in vitro* methodologies and that is the removal of fermentation end products allowing for a longer stable fermentation (Vinyard & Faciola, 2022). The types of CC include the single-flow (SFCC) and dual flow (DFCC) methodologies. The flows of which, refer to the outflow of effluent from the system. In SFCC, the outflow of effluent comes from one single exit (either via overflow of vessel contents or pumped out at a controlled rate) and is a mixture of the solid and liquid fractions of the effluent. One type of SFCC is the rumen simulation technique (RUSITEC). First described by (Czerkawski & Breckenridge, 1977), RUSITEC uses nylon bags of feed within a CC of ruminal fluid that is maintained with constant agitation, the inflow of artificial saliva, and outflow due to overflow; illustrated in (Figure 5), the original RUSITEC design employs the use of an air-tight vessel, making measurement of gas production possible (Martínez et al., 2009; Vinyard & Faciola, 2022).



A, feed within nylon bag;
B, porous nylon bag; **C**, rigid tube used to support bags; **D**, perforated container; **E**, ruminal fluid; **F,** fermentation vessel; **G**, inlet for infusion of artificial saliva; **H,** outlet for digesta removal via overflow; **I**,drive shaft for rotation;
J, sampling port; **K,** airtight rubber seal.

Figure 9 : Schematic diagram of the rumen simulation technique (RUSITEC) system (Czerkawski & Breckenridge, 1977).

The use DFCC was first described by (Hoover et al., 1976). In this system, the outflow is separated into solid and a liquid fraction in which the outflow via overflow is the solid fraction and the filtered, the pumped outflow is the liquid fraction; illustrated in (Figure 6). Thus, the response is more representative of what would be observed *in vivo* than SFCC (Brandao et al., 2020; Brandao & Faciola, 2019). Brandao & Faciola, (2019) examined the variations reported in different DFCC studies, focusing on the influence of dietary composition, particularly CP and NDF as well as daily feed intake on microbial fermentation and end products. They found that estimates of ruminal degradation, VFA concentrations, and N metabolism were consistent across studies utilizing the DFCC system.



A, feed added directly to the ruminal content; **B**, agitator/mixer; **C**, filter for constant removal of liquid fraction; **D,** temperature sensor; **E**, ruminal content; **F**, fermentation vessel; **G**, inlet for infusion of artificial saliva; **H,** outlet for digesta removal via overflow; **I**, drive shaft; **J,** opening for addition of feed/sampling port; **K**, connection to peristaltic pump for liquid removal; **L**, heater.

Figure 10: Schematic diagram of the dual-flow continuous culture system first utilized by (Monteiro et al., 2022).

Both SFCC and DFCC have been used to study changes in the carbohydrate, lipids, proteins, vitamins, and minerals (Del Bianco Benedeti et al., 2015; Ravelo et al., 2022), (Amaral et al., 2016; Paula et al., 2017); (Arce-Cordero et al., 2022); Additionally, these systems have been utilized to assess the effects of various feed additives, such as direct-fed microbials (Monteiro et al., 2022) and exogenous enzymes (Bennett et al., 2021), to evaluate CH_4 emissions (Martínez et al., 2009) and to study the impact of ruminal exposure to toxins (Dai et al., 2019).

As demonstrated by Brandao et al., (2020) and Brandao & Faciola, (2019), Continuous culture (CC) systems, offer a cost- and time-efficient alternative to *in vivo* trials while still providing reliable insights into ruminal fermentation. In the meta-analysis conducted by (Hristov, Callaway, et al., 2012), they comparedSFCC and DFCC with*in vivo* trials to assess variability in ruminal fermentation and nutrient degradation data. They found that DFCC showed the highest variability, followed by RUSITEC, and *in vivo* studies showing the least. However, the continuous culture data did not distinguish between SFCC and DFCC systems, which may have contributed to the variability. Despite being a less costly alternative to *in vivo* trials, continuous culture (CC) systems come with significant initial and maintenance expenses. These costs can outweigh the benefits if the system is not used consistently, making other *in vitro* methods or even *in vivo* trials more cost-effective in some cases (Hristov, et al., 2012). This may explain the limited number of CC systems currently in use. However, when used regularly, a CC system can be reliable, and its maintenance costs can be distributed across multiple experiments, improving overall cost-efficiency. Another detriment of continuous culture (CC) systems is their inability to maintain protozoa at *in vivo* levels, potentially affecting ruminal fermentation. Although protozoal counts are higher in CC than in RUSITEC, they remain lower than *in vivo* due to solid outflow and the low replication rate of protozoa (Hristov et al., 2012; Martínez et al., 2010). Additionally, bacterial, fungal, and methanogens populations also decline during CC fermentation compared to initial levels (Mateos et al., 2017). Despite this, CC systems still produce meaningful and comparable results to *in vivo* studies (Brandao et al., 2020).

3 OBJECTIVES

The objectives of this study were:

1. Identify the most promising feed additives for CH_4 mitigation while maintaining optimal feed fermentation using an *in vitro* batch culture.

2. Assess the most effective combination among the most promising additives selected from Experiments 1 and 2.

3. Evaluate the effects of these combinations over an extended time period using a consecutive batch culure incubations to better simulate the ruminal environment and allow microbial adaptation.

4 MATERIALS AND METHODS

All experimental procedures were carried out under the Project License PI22/25 approved by the Ethic Committee for Animal Experimentation from eh University of Zaragoza, Spain.

4.1 Experiment 1: *In vitro* screening of feed additives

- ***Natural feed additives***

In this experiment, treatments consisted of ten commercial natural feed additives tested at four different doses (Table 2). These additives included garlic extract containing diallyl disulfide as the main active compound, eugenol and fivepolyphenols sources containing gallic acid as the active compound. Moreover, it was evaluated a MIX additive containing the garlic extract (9.9%), eugenol (18%) and polyphenol 10 (72.1%). Additionally, two sources of green seaweed were included.

Table 2:Additives, main active compound, and doses used in Experiment 1

Additive	Active compound	(% in DM)	Active compound dose (mg/g DM)			
			D1	D2	D3	D4
Garlic	Diallyl disulfide	5.65	1.50	3.00	6.00	12.0
Eugenol	Eugenol	15.0	1.00	2.00	4.00	8.00
Polyphenol 1	Gallic Acid	9.96	0.50	1.00	2.00	4.00
Polyphenol 2	Gallic Acid	0.90	0.50	1.00	2.00	4.00
Polyphenol 6	Gallic Acid	5.90	0.50	1.00	2.00	4.00
Polyphenol 7	Gallic Acid	2.60	0.50	1.00	2.00	4.00
Polyphenol 10	Gallic Acid	49.1	0.50	1.00	2.00	4.00
MIX(Gar.Eug.Pol10)	DD+Eug+GA	/	1.81	3.61	7.23	14.5
Green seaweed 1		100	25.0	50.0	100	200
Green seaweed 2		100	25.0	50.0	100	200

The additive dose was calculated according to the % of purity of the main active compound

- ***In vitro rumen fermentation***

Rumen inocula were obtained from four steers slaughtered at a commercial abattoir (Mercazaragoza, Zaragoza, Spain), and rumen contents were collected immediately after evisceration. The rumen fluid from each animal was transported to the laboratory in different airtight thermos flasks, each filtered through two layers of muslin, and diluted (1:4) with an anaerobic incubation buffer adjusted to pH 6.8 according to (McDougall, 1948) to mimic rumen environmental conditions in dairy cows.
Batch culture incubations(Theodorou et al., 1994)were conducted in 125 mL Wheaton bottles containing 50 mL of buffered rumen inoculum and 0.5 g DM of a substrate (60:40 forage:concentrate), formulated to meet the nutritional requirements of a dairy cow producing 36 kg of milk at 12 weeks of lactation. The substate in (g/kgDM) consisted in tall fescue hay (400), alfalfa hay (200), soybean hulls (140), wheat (80), barley (80),, corn (80), and soybean meal (20).The substrates were chemically analysed according to AOAC, (2005) and (Van Soest et al., 1991). The chemical composition of the substrate (g/kg DM) was as follows: organic matter (OM,935), crude protein (CP,146), ether extract (EE,19.3), neutral detergent fiber (NDF,398), acid detergent fiber (ADF,227), and acid detergent lignin

(ADL,42.8). Feed additives were top-added to the diet given their low inclusion rate. This inclusion rate was calculated to provide the same amount of each active compound within each category. The seaweeds were incorporated in the diet by replacing the fescue hay due to their higher inclusion rate. Each feed additive was tested at four concentrations as described in Table 2 with four experimental replicates per dose. Additional bottles without additives were included as Control and without substrate as blanks for each rumen inoculum.

Bottles were incubated anaerobically at 39°C for 24 h. GP was measured at 3, 10, and 24 h using a HD 2124.02 manometer fitted with a TP804 pressure gauge (Delta Ohm, Caselle di Selvazzano, Italy), and pressure readings were converted to volume (mL) 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 10–24 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 meter 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.

CH_4 concentration in the fermentation gas was measured using gas chromatography apparatus (Agilent 6890 Series GC System, Santa Clara, USA) equipped with a FID detector and a capillary column (HP-1, 30 m × 535 µm). The VFA concentration was determined by gas chromatography on the same apparatus as for the CH_4, with a capillary column (HP-FFAP Polyethylene glycol TPA, 30 m × 530 µm) as described by(Jouany, 1982). The molar proportion of branched-chain volatile fatty acids (BCVFA) was calculated as the sum of iso-butyrate and iso-valerate and used as an indicator of protein breakdown, as these compounds are key products of valine and leucine degradation, respectively. The concentration of NH_3-N was determined colorimetricallyas described by(Chaney & Marbach, 1962).

- ***Statistical analyses***

All statistical analyses were conducted using SPSS software (IBM Corp., Version 29.0, New York, USA). The minimum effective dose, defined as the lowest concentration at which a given additive exhibited a significant difference compared to the control, was determined through analysis of variance (ANOVA) using the following statistical model:

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

where Y_{ijk} is the dependent, continuous variable, μ is the overall population mean, D_i is the fixed effect of the dose of each additive *(i*=CTL vs D1 vs D2 vs D3 vs D4), A_j is the random effect (j = 1 to 4) of the animal as rumen inoculums e_{ijk} is the residual error. When significant effects of doses were detected, means were compared using LSD test. Additionally, linear (L) and quadratic (Q) orthogonal contrasts were performed to evaluate the dose-response effect for each feed additive.

4.2 Experiment 2: Short and long-term effects of combinations of additives from Experiment 1

A second *in vitro* experiment was conducted to evaluate the short- and long-term effects of combining the three most promising additives from Experiment 1: garlic powder (G), eugenol (E), polyphenol 6 extract (P) rich in gallic acid. These additives were selected for their ability to reduce CH_4 emissions without compromising rumen fermentation. Each additive was tested alone and in combination of the other in order to identify potentialsynergistic effects as detailed in Table 3. Target doses were calculated based on the most effective concentration of their main active compounds.

Table 3: Treatments evaluated in Experiment 2

Treatments	Garlic	Eugenol	Polyphenol
G100	100%		
E100		100%	
P100			100%
G50-E50	50%	50%	
G50-P50	50%		50%
E50-P50		50%	50%
G75-E12-P12	75%	12.5%	12.5%
G12-E75-P12	12.5%	75%	12.5%
G12-E12-P75	12.5%	12.5%	75%
E33-P33-G33	33%	33%	33%

E100 = 60 mg eugenol/gDM; P100 = 3 mg gallic acid/g DM; G100 = 12 mg diallyl disulfide/g DM

- ***Consecutive Batch culture***

Incubations were carried out following the procedure described in Experiment 1, aiming to assess both short-term (1-day) and long-term (5-day) effects of the additives on GP, CH_4emissions, and fermentation parameters. A total of 48 Wheaton bottles were used over a five-day periodto test all additive combinations, along with two control treatments without additives to represent a dose zero (D0) equivalent to of 0 mg/gDM. Each treatment was performed in quadruplicate using ruminal fluid obtained from four cattle slaughtered at a commercial abattoir (Mercazaragoza, Zaragoza, Spain) as described in Experiment 1.

On day 1, ruminal fluid from each animal was mixed with buffer solution (1:4) under anaerobic conditions, and 50 mL of this mixture was dispensed into each Wheaton bottle. The bottles were then sealed and incubated in a water bath at 39 °C. The substrates were identical to experiment1.GP was measured at 3, 10, and 24 hours, with gas samples collected at the same time points using Vacutainers, as previously described for experiment 1.After 24 hours of incubation, bottles were opened, and 12.5 mL of fermentation fluid from each bottle was transferred into new bottles containing 37.5 mL of buffer solution withnewsubstrate (0.5 g DM) and the corresponding additive treatment, initiating the second incubation cycle. This procedure was repeated during 5 consecutive days, with incubation cycles refreshed daily. During days 2, 3 and 4, the GP at 3, 10 and 24h were recorded and gas was released, moreover incubation pH was measured every day after 24h of incubation. On day 1 and 5, bottles were open after 24h of incubation, pH was recorded, and 1 mL samples were collected for VFA and 1 mL for ammonia-N analyses. During these sampling days, total GP and CH_4 concentration were also measured at 3, 10 and 24h of incubation.

- ***Statistical analyses***:

Data were analysed by a repeated measures analysis using the SPSS (IBM Corp., Version 27.0, New York, USA) as follows:

$$\boldsymbol{Y_{ijk} = \mu + A_i + D_j + AD_{ij} + R_k + B_l + e_{ijklm}}$$

where Y_{ijk} is the dependent, continuous variable, μ is the overall population mean, A_i is the fixed effect of the additive (i = CTL vs G100 vs E100 vs P100 vs G50-E50 vs G50-P50 vs E50-P50 vs G75-E12-P12 vs G12-E75-P12 vs E12-P12-G75 vs E33-P33-G33), D_j is the fixed effect of the day (j = 1 vs 5), AD_{ij} is the interaction term, R_j is the random effect (j = 1 to 4) of the animal used as rumen inoculum, $B_l$is the random effect of the incubation bottle (l = 1 to 44) and $e_{ijk}$is the residual error.When significant effects of doses were detected, means were compared using LSD test.

Given that the interaction between the additive and the day was significant for most parameter analysed, a second analysis was conducted to facilitate the interpretation of this interaction. This ANOVA investigated the effects of the additives for each incubation day as follows:

$$\boldsymbol{Y_{ijk} = \mu + A_i + R_j + e_{ijk}}$$

where Y_{ijk} is the dependent, continuous variable, μ is the overall population mean, A_i is the fixed effect of the additive (i = CTL vs E100 vs P100 vs G100 vs E50-P50 vs E50-G50 vs P50-G50 vs E75-P-12-G12 vs E12-P75-G12 vs E12-P12-G75 vs E33-P33-G33), R_j is the random effect (j = 1 to 4) of the animal used as rumen inoculum and $e_{ijk}$is the residual error.Results were considered significant at $P< 0.05$, and trends were discussed at $P< 0.1$.

4.3 Experiment 3: *In vitro* screening of feed additives at higher doses

Since some additives used in Experiment 1 showed no significant effects, a second screening was conducted using higher doses of Garlic, Polyphenol 6, and MIX, along with eight new additives provided by CCPA-Group (Table 4). As in Experiment 1, the objective was to evaluate dose–response effects on rumen fermentation and CH_4 production, testing four different inclusion levels for each additive.

Table 4: Feed additives, their main active compound, and doses used in Experiment 3

Additive	**Active compound**	(%)	**Active compound dose (mg/g DM)**			
			D1	D2	D3	D4
Garlic 1	Diallyldisulfide	5.65	1.50	3.00	6.00	12.0
Garlic 2	Diallyldisulfide	4.87	1.50	3.00	6.00	12.0
MIX	Gar.+Eug.+Pol 5		11.0	22.0	44.0	88.0
Polyphenol 6	Gallic Acid	5.9	1.50	3.00	6.00	12.00
Polyphenol 12	Gallic Acid	100	0.15	0.30	0.60	1.20
Polyphenol 13	Gallic Acid	9.0	0.15	0.30	0.60	1.20
Polyphenol 14	Gallic Acid	30	0.15	0.30	0.60	1.20
Cinnamon	Essential oils	100	25.0	50.0	100	200
Oregano	Essential oils	100	25.0	50.0	100	200
BES	BES	>97	0.05	0.10	0.20	0.40

To ensure uniform solvent exposure across treatments, certain additives (Polyphenol 12, Cinnamon, and Oregano) were diluted in ethanol, while BES was diluted in water, depending on their solubility and stability. To maintain standardized conditions, an equivalent amount of ethanol was added to all treatments. Fermentation and sampling procedures were conducted as previously described in Experiment 1. An analysis of variance was performed as in Experiment 1 to identify the minimum effective dose, defined as the lowest concentration at which a given additive produced a statistically significant effect compared to the control.

4.4 Experiment 4: Short and long-term effects of combinations of additives from Experiment 2

A fourth *in vitro* experiment was carried out to evaluate the effects of combining the three most promising additives from Experiment 3: Garlic1 and two essential oils; Cinnamon and Oregano. These additives were selected based on their capacity to decrease CH_4 emissions without negatively impacting rumen fermentation; each additive was tested individually and in various combination proportions, as described in Table 5. Doses were calculated based on the concentration of their main active compounds.

The incubation was conducted during 5 consecutive days and fermentation conditions and sampling during day 1 and 5 were consistent with those previously described experiments 2. Data were analysed by a repeated measures analysis and ANOVA using the SPSS (IBM Corp., Version 27.0, New York, USA) as described in experiment 2.

Table 5 : Treatments evaluated in experiment 4

Treatments	Garlic1	Cinnamon	Oregano
$G_1$100	100%		
C100		100%	
O100			100%
$G_1$50-C50	50%	50%	
$G_1$50-O50	50%		50%
C50-O50		50%	50%
$G_1$75-C12-O12	75%	12.5%	12.5%
$G_1$12-C75-O12	12.5%	75%	12.5%
$G_1$12-C12-O75	12.5%	12.5%	75%
$G_1$33-C33-O33	33%	33%	33%
$G_1$100=12mg diallyldisulfide/gDM; C100=80mg/gDM; O100= 50mg/gDM			

4.5 Experiment 5: RUSITEC Calibration Trial

In the final phase of the internship, two Rumen Simulation Technique (RUSITEC) systems were newly installed in the laboratory. As part of the system setup and validation, a preliminary trial was conducted with the aim of calibrating the system and ensuring its functionality prior to further experimental applications.

- ***System setup and peristaltic pump calibration***

The RUSITEC system was assembled in accordance with the manufacturer's instructions. Each fermentation vessel was inspected to ensure proper functionality and structural integrity. All tubing connections were verified and appropriately assigned: from the peristaltic pump to the fermenters, from the fermenters to the overflow vessels, and from the overflow vessels to the gas collection bags.

The peristaltic pump responsible for buffer infusion was calibrated to deliver a flow rate of 860 mL/day, simulating *in vivo* salivary secretion and corresponding to a dilution rate of approximately 3.5%/h. Calibration was performed by adjusting the pressure applied by the pump head and taking into account the tubing length, internal diameter, and flow resistance.

- ***Rusitec technique***

Each Rusitec machine consisted of eight (1100-mL) vessels immersed in a water bath maintained at 39ºC and provided with permanent vertical agitation. Two Rusitec machines were used in the study to give 16 vessels in total. Experimental diets were considered in order to generate differences across treatments: a high foraged diet (FOR) was made of 80% fescue hay and 20% concentrate whereas a high concentrate diet (CON) was made of 80% concentrate and 20% fescue hay. Diets were allocated alternatively to the vessels (eight vessels per treatment) having four treatment replicates (two from each source) in each Rusitec machine. Rumen fluids and solids were obtained from six rumen-cannulated ewes fed at maintenance level (80% tall fescue hay and alfalfa hay and 20% concentrate on DM basis) and managed according to the protocols approved by the Ethic Committee for Animal Experimentation from the University of Zaragoza, Spain. Rumen contents were collected immediately before the morning feeding, strained through a double layer of muslin and transferred to the laboratory in six thermo flasks.

In the laboratory, the six rumen fluids were pooled into a single homogenized inoculum, which was then used to inoculate all vessels across the two RUSITEC machines. This pooling was conducted in order to test the repeatability across fermenters. On Day 1 of the experiment, each vessel was inoculated with 380 mL of rumen fluid, 770 mL of artificial saliva (McDougall, 1948), each nylon bag containing 20g of rumen solid digesta and another containing 20g of the forage of interest. Artificial saliva was prepared daily, adjusted to pH 6.8 and continuously infused at a rate of 860 mL/day (dilution rate of 0.036%/h) using a peristaltic pump. The displaced effluent and fermentation gasses from each fermentation vessels were collected into effluent bottles containing 20 ml of H2SO4 at 20% vol/vol to stop the microbial fermentation. Daily GP was collected in bags and the volume was measured using a gas meter (Ritter, TG/1/5-50) mbar equipment. After 24 h, each vessel was opened; one of the initial two bags containing rumen digesta solids was removed, squeezed and washed with 30 ml of artificial saliva. The liquid fractions of the washings were returned to the vessels and a new nylon bag was inserted containing new diet. On the following days, the nylon bag that had been incubated in the vessel for 48 hours was replaced with a new bag containing plant material, as previously described. The incubation run lasted for five days, during which daily measurements of overflow volume and GP were recorded. No samples for VFA or Ammonia-N were collected, as the objective of the experiment was solely to evaluate the system's Functionality and setup. The digestibility of organic matter (OM) was determined by calculating the weight loss of nylon bags after 48 hours of incubation. Following incubation, the bags were thoroughly rinsed, then dried at 60 °C for 48 hours before weighing.

Figure 11: Rusitec system at the laboratory of Animal production at the faculty of Veterinay (University of Zaragoza).

5 RESULTS

5.1 Experiment 1: *In vitro* Batch Incubations

Dietary supplementation with increasing doses of four classes of bioactive additives; namely Polyphenols (0–4 mg/g DM), Garlic extract (0–12 mg/g DM), Eugenol (0–80 mg/g DM) and Green-Seaweeds (0–200 mg/g DM) revealed distinct, dose-dependent effects on *in vitro* ruminal fermentation. In polyphenols group (Tables 6 and 7): Increasing Polyphenol 1 supplementation had no impact on any fermentation parameter analyzed or CH_4 production. Polyphenol 2 linearly increased total GPand linearly decreased pH and NH_3-N concentration. Polyphenol 7 induced strong linear decreases in pH ($P < 0.01$) and NH_3–N ($P < 0.001$) without affecting VFA concentrations. Increasing supplementation with polyphenol 7 promoted a linear increment in the total GP and ultimately the total CH_4 production as the percentage of CH_4 remained constant. Increasing supplementation with Polyphenol 10 progressively lowered total VFA concentration ($P < 0.05$) while linearly increased butyrate molar proportion ($P < 0.05$) without affecting the rest of the parameters considered.

Table 6*:* Effect of increasing doses of polyphenols1 and 2 used in experiment 1on *in vitro* gas production and fermentation characteristics.

	Active compound dose (mg/gDM)							
	0	**0.5**	**1**	**2**	**4**	SEM	P-value	Contrast
Polyphenol 1								
pH	6.73	6.73	6.74	6.74	6.73	0.008	NS	NS
NH3-N(mg/dL)	31.2	30.7	30.5	30.9	31.1	0.277	NS	NS
Total VFA (mmol/L)	66.5	53.6	79.7	62.7	67.9	13.19	NS	NS
Acetate (%)	59.4	53.4	58.8	60.0	55.2	5.310	NS	NS
Propionate (%)	20.5	21.8	21.6	19.4	22.7	2.112	NS	NS
Butyrate (%)	13.4	16.1	13.5	13.5	15.1	2.028	NS	NS
Valerate(%)	1.57	1.97	1.42	1.41	1.58	0.287	NS	NS
BCFA.(%)	5.12	6.67	4.68	5.71	5.43	1.100	NS	NS
A/P Ratio	2.98	2.47	2.80	4.40	2.51	1.037	NS	NS
Total GP (mL)	91.3	93.7	90.8	90.2	92.5	1.113	NS	NS
CH_4 (%)	3.24	3.44	3.46	3.4	3.38	0.126	NS	NS
Total CH_4 (mL)	3.10	3.37	3.31	3.19	3.27	0.257	NS	NS
CH_4/VFA (mL/mol)	49.6	73.2	43.5	64.4	54.3	13.04	NS	NS
Polyphenol 2								
pH	6.73	6.72	6.71	6.72	6.70	0.009	NS	L^{*}
NH3-N(mg/dL)	31.2	30.6	30.5	30.5	29.8	0.338	NS	L^{*}
Total VFA (mmol/L)	66.5	60.2	58.0	59.2	67.0	8.220	NS	NS
Acetate (%)	59.4	61.5	56.6	55.3	57.6	3.490	NS	NS
Propionate (%)	20.5	19.5	22.2	23.0	21.7	1.605	NS	NS
Butyrate (%)	13.4	12.8	14.4	14.7	14.2	1.294	NS	NS
Valerate(%)	1.57	1.29	1.54	1.57	1.64	0.164	NS	NS
BCFA.(%)	5.12	4.89	5.31	5.40	4.90	0.578	NS	NS
A/P Ratio	2.98	3.51	2.61	2.41	2.68	0.496	NS	NS
Total GP (mL)	91.3^{a}	93.3^{a}	93.1^{a}	93.8^{a}	97.5^{b}	1.102	*	L^{**}
CH_4 (%)	3.24	3.56	3.58	3.48	3.51	0.138	NS	NS
Total CH_4 (mL)	3.10	3.47	3.48	3.40	3.59	0.141	NS	L^{T}
CH_4/VFA (mL/mol)	49.6	58.6	63.2	59.6	58.5	8.680	NS	NS

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; L. linear response; Q. quadratic response. ***. P<0.001; **. P<0.01; *. P<0.05; T. P<0.1; NS. P>0.1

Table 7: Effect of increasing doses of polyphenols 7 and 10 used in experiment 1on *in vitro* gas production and fermentation characteristics.

	Active compound dose (mg/gDM)							
	0	**0.5**	**1**	**2**	**4**	SEM	P-Value	Contrast
Polyphenol 7								
pH	6.73^{b}	6.71^{b}	6.71^{b}	6.70^{b}	6.67^{a}	0.008	**	L^{***}
NH3-N(mg/dL)	31.2^{c}	30.1^{b}	30.5^{b}	28.8^{a}	28.5^{a}	0.287	***	$L^{***}.Q^{*}$
Total VFA (mmol/L)	66.5	62.4	58.7	62.5	64.2	8.210	NS	NS
Acetate (%)	59.4	58.2	55.8	56.4	56.1	3.050	NS	NS
Propionate (%)	20.5	21.8	22.7	23.3	23.6	1.251	NS	NS
Butyrate (%)	13.4	13.6	14.5	14.1	14.1	1.144	NS	NS
Valerate(%)	1.57	1.70	1.85	1.76	1.66	0.197	NS	NS
BCFA.(%)	5.12	4.61	5.10	4.43	4.54	0.711	NS	NS
A/P Ratio	2.98	2.69	2.53	2.45	2.44	0.305	NS	NS
Total GP (mL)	91.3^{a}	91.9^{a}	94.4^{a}	99.8^{b}	108^{c}	1.443	***	L***
CH_4 (%)	3.24	3	3.54	3.63	3.72	0.26	NS	NS
Total CH_4 (mL)	3.10^{ab}	2.85^{a}	3.49^{abc}	3.73^{bc}	4.12^{c}	0.268	*	L**
CH_4/VFA (mL/mol)	49.6	46.0	63.6	61.1	73.3	9.900	NS	LT
Polyphenol 10								
pH	6.73	6.71	6.73	6.71	6.71	0.011	NS	NS
NH3-N(mg/dL)	31.2	31.2	31.4	31.1	30.9	0.372	NS	NS
Total VFA (mmol/L)	66.5^{b}	65.9^{b}	59.1^{ab}	55.6^{ab}	46.7^{a}	4.410	*	L**
Acetate (%)	59.4	58.1	56.4	55.0	53.1	2.580	NS	LT
Propionate (%)	20.5	22.7	22.8	23.4	23.3	1.244	NS	NS
Butyrate (%)	13.4	13.4	14.5	15.1	16.1	0.928	NS	L*
Valerate(%)	1.57	1.47	1.58	1.59	1.80	0.118	NS	LT
BCFA.(%)	5.12	4.37	4.71	4.95	5.72	0.481	NS	NS
A/P Ratio	2.98	2.60	2.50	2.44	2.28	0.268	NS	NS
Total GP (mL)	91.3	93.8	91.2	92.4	91.7	1.383	NS	NS
CH_4 (%)	3.24	3.67	3.68	3.56	3.49	0.129	NS	NS
Total CH_4 (mL)	3.10	3.59	3.47	3.42	3.35	0.123	NS	NS

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; L. linear response; Q. quadratic response. ***, P<0.001; **, P<0.01; *, P<0.05; T, P<0.1; NS. P>0.1

Garlic extract exerted the strongest overall effects (Table 8), markedly lowering pH, NH_3–N concentration and the acetate:propionate ratio (all $P < 0.001$), shifting VFA synthesis from acetate toward propionate ($P < 0.01$) and butyrate ($P < 0.01$) and drastically suppressing CH_4 production ($P < 0.001$). Moreover, these effects occurredwithoutinhibiting feed fermentation as total VFA concentration and total GP remained constant with increasing doses of garlic supplementation. By contrast, eugenol supplementation up to 80 mg/g DM promoted minor effects on the rumen fermentation with no significant changes in pH, NH_3-N, VFAs or total GP. Only a CH_4 increase production ($P < 0.05$) was noted with increasing doses of eugenol supplementation, as well as a tendency to a quadratic decrease in butyrate and BCVFA molar proportions when supplemented at 40 mg/g DM.

Table 8: Effect of increasing doses of Garlic and Eugenol used in experiment 1 on *in vitro* gas production and fermentation characteristics.

	Active compound dose (mg/gDM)					SEM	P-value	Contrast
Garlic	**0**	**1.5**	**3**	**6**	**12**			
pH	6.73^{c}	6.71^{c}	6.65^{b}	6.63^{b}	6.59^{a}	0.008	***	$L^{***}.Q^{**}$
NH3-N(mg/dL)	31.2^{c}	30.7^{c}	28.7^{bc}	26.7^{b}	23.7^{a}	0.856	***	L^{***}
Total VFA (mmol/L)	66.5	74.6	70.5	58.1	59.3	6.170	NS	NS
Acetate (%)	59.4^{c}	56.7^{c}	49.6^{b}	45.3^{ab}	40.5^{a}	2.113	***	$L^{***}.Q^{*}$
Propionate (%)	20.5^{a}	22.9^{a}	27.0^{b}	30.4^{b}	34.5^{c}	1.186	***	$L^{***}.Q^{*}$
Butyrate (%)	13.4^{a}	14.2^{ab}	16.7^{bc}	17.8^{c}	19.0^{c}	0.870	**	$L^{***}.Q^{T}$
Valerate(%)	1.57	1.48	1.69	1.78	2.11	0.150	T	L^{**}
BCFA.(%)	5.12	4.68	5.04	4.71	3.88	0.476	NS	L^{T}
A/P Ratio	2.98^{c}	2.50^{bc}	1.85^{ab}	1.50^{a}	1.19^{a}	0.219	***	$L^{***}.Q^{*}$
Total GP (mL)	91.3^{a}	93.8^{a}	92.8^{a}	94.4^{a}	101^{b}	2.015	*	$L^{**}.Q^{**}$
CH_4 (%)	3.25^{d}	3.08^{d}	1.75^{c}	0.91^{b}	0.47^{a}	0.11	***	$L^{***}.Q^{***}$
Total CH_4 (mL)	3.10^{d}	3.00^{d}	1.67^{c}	0.89^{b}	0.52^{a}	0.113	***	$L^{***}.Q^{***}$
CH_4/VFA (mL/mol)	49.6^{c}	42.1^{c}	24.9^{b}	15.1^{ab}	8.12^{a}	4.410	***	$L^{***}.Q^{**}$
Eugenol	**0**	**1**	**2**	**4**	**8**			
pH	6.73	6.73	6.72	6.73	6.73	0.008	NS	NS
NH3-N(mg/dL)	31.2	29.6	30.1	30.6	30.8	0.584	NS	NS
Total VFA (mmol/L)	66.5	59.9	70.1	74.2	59.4	4.450	NS	Q^{T}
Acetate (%)	59.4	56.0	59.7	60.1	55.3	1.947	NS	NS
Propionate (%)	20.5	23.4	21.4	21.4	22.7	0.967	NS	NS
Butyrate (%)	13.4	14.4	13.1	12.9	15.3	0.681	NS	Q^{T}
Valerate(%)	1.57	1.46	1.49	1.41	1.63	0.101	NS	NS
BCFA.(%)	5.12	4.71	4.25	4.11	5.04	0.422	NS	QT
A/P Ratio	2.98	2.41	2.82	2.83	2.44	0.232	NS	NS
Total GP (mL)	91.3	91.5	90.5	90.4	91.2	0.7600	NS	NS
CH_4 (%)	3.24	3.45	3.43	3.55	3.56	0.13	NS	NS
Total CH_4 (mL)	3.10	3.30	3.29	3.37	3.51	0.1138	NS	L^{*}
CH_4/VFA (mL/mol)	49.6	55.4	49.4	46.9	63.4	4.450	NS	L^{T}

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; L. linear response; Q. quadratic response. ***. P<0.001; **. P<0.01; *. P<0.05; T. P<0.1; NS. P>0.1

Similarly, to the other polyphenols studied, Polyphenol 6 also caused a linearpH decline without altering the fermentation pattern. This polyphenol promoted a linear increase in the total CH_4 production which was associated to tendency to linearly increase total GP.MIX is a combination formed by (10%Garlic, 20% Eugenol and 70% polyphnol 6) when supplemented up to 4 mg/g DM promoted a strong linear decrease in NH_3-N concentration ($P < 0.001$) as well as a tendency to linearly decrease total VFA concentration. MIX supplementation also promoted a shift in the VFA production characterized by a linear decrease in the acetate and linear increases in propionate and butyrate molar proportions which led to a linear decrease in the A/P ratio. MIX supplementation did modify neither total GP nor CH production when tested at these doses (Table 9).

Table 9: Effect of increasing doses of Polyphenol 6 and MIX used in experiment 1 on *in vitro* gas production and fermentation characteristics.

	Active compound dose (mg/gDM)					SEM	P-Value	Contrast
Polyphenol 6	**0**	**0.5**	**1**	**2**	**4**			
pH	6.73^{b}	6.74^{b}	6.72^{ab}	6.72^{ab}	6.70^{a}	0.006	*	L^{**}
NH3-N(mg/dL)	31.2	30.7	31.2	31.7	31.6	0.478	NS	NS
Total VFA (mmol/L)	66.5	66.9	64.0	58.4	54.6	8.110	NS	NS
Acetate (%)	59.4	57.0	54.2	56.4	56.0	2.290	NS	NS
Propionate (%)	20.5	22.7	23.7	22.4	22.8	1.128	NS	NS
Butyrate (%)	13.4	14.2	15.3	14.6	14.7	0.780	NS	NS
Valerate(%)	1.57	1.51	1.69	1.63	1.58	0.103	NS	NS
BCFA.(%)	5.12	4.60	5.13	5.04	5.00	0.515	NS	NS
A/P Ratio	2.98	2.55	2.33	2.53	2.47	0.252	NS	NS
Total GP (mL)	91.3	93.6	93.2	93.6	94.8	0.999	NS	L^{T}
CH_4 (%)	3.24	3.34	3.54	3.5	3.65	0.13	NS	NS
Total CH_4 (mL)	3.10	3.30	3.41	3.42	3.60	0.125	NS	L^{*}
CH_4/VFA (mL/mol)	49.6	49.6	59.6	59.4	66.0	6.000	NS	L^{T}
MIX	**0**	**5**	**10**	**20**	**40**			
pH	6.73	6.68	6.72	6.71	6.70	0.014	NS	NS
NH3-N(mg/dL)	31.2^{c}	30.3^{bc}	30.7^{c}	29.3^{ab}	28.4^{a}	0.416	***	L^{***}
Total VFA (mmol/L)	66.5	64.4	63.1	55.8	49.3	6.740	NS	L^{T}
Acetate (%)	59.4	60.1	57.8	56.9	51.5	2.610	NS	L^{*}
Propionate (%)	20.5	21.1	22.4	22.6	25.1	1.171	NS	L^{*}
Butyrate (%)	13.4	13.0	13.8	14.1	16.1	0.960	NS	L^{*}
Valerate(%)	1.57	1.59	1.37	1.60	1.72	0.159	NS	NS
BCFA.(%)	5.12	4.27	4.59	4.82	5.56	0.506	NS	NS
A/P Ratio	2.98	2.84	2.63	2.61	2.11	0.250	NS	L^{*}
Total GP (mL)	91.3	91.0	93.0	90.1	93.0	1.554	NS	NS
CH_4 (%)	3.24	3.31	3.43	3.63	3.31	0.125	NS	NS
Total CH_4 (mL)	3.10	3.15	3.30	3.45	3.20	0.149	NS	NS
CH_4/VFA (mL/mol)	49.6	49.2	54.8	67.0	70.6	8.290	NS	L^{*}

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; L. linear response; Q. quadratic response. ***, P<0.001; **, P<0.01; *, P<0.05; T, P<0.1; NS. P>0.1

Supplementation with green seaweed 1elicited a linear rise in NH_3–N concentration ($P < 0.05$). As well as the butyrate and valerate molar proportions ($P < 0.01$) whereas acetate proportion linearly decreased ($P < 0.05$). Green seaweed 1 did not promoted changes in the total GP or CH_4 production when tested at increasing doses. Green seaweed 2 produced a highly significant linear increase in NH_3–N ($P < 0.001$) with a modest linear valerate increase. Seaweed 2 supplementation promoted a small decreased in pH and tended to increase CH_4 production when supplemented at100 mg/g without affecting GP or VFA molar proportions as described in (Table 10).

Table 10: Effect of increasing doses of seaweeds used in experiment 1 on *in vitro* gas production and fermentation characteristics

	Active compound dose (mg/gDM)							
	0	**25**	**50**	**100**	**200**	SEM	P-value	Contrast
Green-Seaweed 1								
pH	6.73	6.72	6.74	6.73	6.75	0.014	NS	NS
NH3-N(mg/dL)	31.2^{a}	32.2^{ab}	33.9^{bc}	33.7^{bc}	35.6^{c}	0.746	*	L^{**}
Total VFA (mmol/L)	66.5	76.8	67.2	62.2	62.2	6.280	NS	NS
Acetate (%)	59.4	60.1	57.3	54.1	55.0	1.880	NS	L^{*}
Propionate (%)	20.5	21.4	22.2	23.2	22.0	0.890	NS	Q^{T}
Butyrate (%)	13.4	12.9	14.2	15.3	15.0	0.662	NS	L^{*}
Valerate(%)	1.57^{a}	1.67a	1.77^{ab}	2.06^{bc}	2.22^{c}	0.105	**	L^{***}
BCFA.(%)	5.12	4.05	4.58	5.38	5.77	0.467	NS	L^{T}
A/P Ratio	2.98	2.84	2.59	2.37	2.50	0.216	NS	NS
Total GP (mL)	91.3	94.1	89.8	93.8	88.8	3.420	NS	NS
CH_4 (%)	3.24	3.62	3.75	3.46	3.51	0.15	NS	NS
Total CH_4 (mL)	3.10	3.67	3.65	3.43	3.27	0.280	NS	NS
CH_4/VFA (mL/mol)	49.6	50.4	54.4	62.4	58.9	5.550	NS	NS
Green-Seaweed 2								
pH	6.73	6.72	6.71	6.70	6.74	0.0114	NS	Q^{T}
NH3-N(mg/dL)	31.2^{a}	32.2^{a}	32.8^{ab}	34.0^{b}	37.2^{c}	0.523	***	L^{***}
Total VFA (mmol/L)	66.5	67.5	73.5	60.3	64.5	9.64	NS	NS
Acetate (%)	59.4	56.3	56.2	58.9	56.3	4.42	NS	NS
Propionate (%)	20.5	22.7	22.8	20.4	21.3	2.074	NS	NS
Butyrate (%)	13.4	14.5	14.3	13.8	14.6	1.542	NS	NS
Valerate(%)	1.57	1.73	1.89	1.84	2.47	0.240	NS	L^{*}
BCFA.(%)	5.12	4.66	4.73	5.04	5.30	0.723	NS	NS
A/P Ratio	2.98	2.49	2.83	2.91	2.82	0.448	NS	NS
Total GP (mL)	91.3	92.6	89.6	94.4	92.4	1.805	NS	NS
CH_4 (%)	3.24	3.53	3.52	3.72	3.62	0.114	NS	NS
Total CH_4 (mL)	3.10	3.45	3.29	3.70	3.52	0.137	T	L^{T}. Q^{T}
CH_4/VFA (mL/mol)	49.6	56.4	53.2	62.3	59.8	7.95	NS	NS

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; L. linear response; Q. quadratic response.
***. P<0.001; **. P<0.01; *. P<0.05; T. P<0.1; NS. P>0.1

5.2 Experiment 2: Short- and long-term incubation of various combinations of additives from Experiment1

- ***In vitro GP over five daysof consecutive incubations***

Over the course of the 5-days consecutive incubations, all treatments exhibited a pronounced drop in GP between day 1 and day 2 (Figure 8), which is consistent with an initial microbial adaptation phase then followed by a partial recovery toward day 5. GP in the control (CTL) dropped initially by 21%, then stabilized on days 3 and 4, and finally recovered by day 5, registering only 12% decrease compared to day 1. By contrast, garlic alone (G100) at 12 mg/g DM maintained a higher overall GP. Although it exhibited an initial decline similar to the CTL, recovery occurred by from day 4, and by day 5 GP exceeded the initial level, which may indicate a potential stimulatory effect on fermentative activity. The polyphenol alone (P100) began with the highest GP (+5% compared to CTL) and followed a similar slump–rebound trajectory thereafter. Eugenol alone (E100) exhibited the strongest suppression, with GP dropping to (-42%) by day 3, and only partially recovering to (-29%) by day 5. Notably, the garlic-blend treatments showed a much more modest initial decline and then recovered by day 5 near toinitialGP, underscoring garlic's superior ability to sustain fermentative activity. In contrast, blends dominated by polyphenol (P75–G12–E12) closely mirrored the CTL kinetics, while the Eugenol-dominated blend (E75–G12–P12) showed a similar but attenuated response compared with E100. These observations emphasize the time-dependent nature of ruminal adaptation.

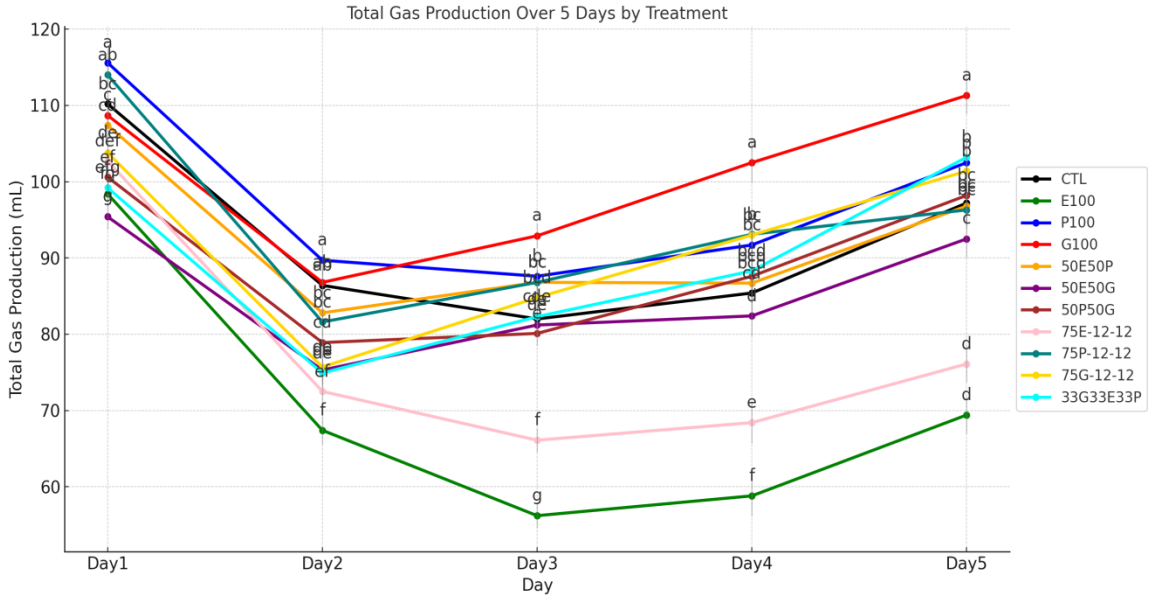


Figure 12 : Gas production over 5 days *in vitro* incubation with Garlic, Eugenol, Polyphenol and their combinations

- ***Short term effect on CH_4 production and fermentation characteristics***

Supplementation with garlic alone (G100 at 12 mg/g DM) reduced total CH_4 production by 78% ($P < 0.001$) without affecting pH or total VFA concentration, and concurrently decreased NH_3–N levels by 16% ($P < 0.001$). Garlic significantly altered VFA stoichiometry, with acetate decreasing by 12%, propionate increasing by 31%, and butyrate by 25%, leading to a pronounced reduction in the acetate:

propionate ratio (all P < 0.001). In contrast, eugenol alone (E100) did not affect CH_4, pH, or total VFA, but slightly modified the VFA profile by increasing acetate by 3% and decreasing propionate by 13% (P < 0.001). The polyphenol alone (P100) slightly increased total GP (+5%) and CH_4 production (+9%), (Both P < 0.001), without altering total or individual VFA concentrations.

Binary blends containing 50% garlic (G50–E50 and G50–P50) retained most of garlic antimethanogenic activity, reducing CH4 production by approximately 70% compared to the control. Both blends also resulted in NH_3–N levels lower than the control but higher than those observed with pure garlic (G100). Among them, (G50–P50) induced the greatest reduction in total VFA production (-12%), suggesting that polyphenols may attenuate garlic's stimulatory effect on fermentation observed in pure garlic. The G50–E50 blend also reduced VFA concentrations, but the difference was not statistically significant when compared to either CTL or G100. Conversely, the E50–P50 combination behaved similarly to eugenol alone. Ternary mixtures revealed a dose-dependent effect of garlic: (G75–E12–P12) blend achieved the greatest reduction in CH_4 (-75%), followed by (G33–E33–P33) with (-63%), while blends dominated by eugenol or polyphenols, such as (E75–G12–P12) and (P75–G12–E12), were less effective (-24% and -6%, respectively).In contrast, the polyphenol-rich blend (P75–G12–E12) yielded the highest VFA production All garlic-based blends preserved garlic's propionigenic and butyrogenic effects. For instance, the (G75–E12–P12) blend induced a 25% increase in propionate and a 12% decrease in acetate. A similar shift was observed in the (G33–E33–P33) blend (Table 11 and 12).

Table 11: Overall fermentation parameters after 1-day *in vitro* fermentation with Garlic, Eugenol, Polyphenol and their combinations

	Total GP (ml)	CH_4 (%)	Total CH_4 (ml)	pH	NH_3-N (mg/dl)	VFAs (mmol/L)	CH_4:VFAs
CTL	110^{bc}	5.14^{ab}	5.67^{ab}	6.58	35.4^{ab}	89.3^{abc}	63.7^{ab}
G100	108^{c}	1.10^{e}	1.21^{f}	6.57	29.6^{d}	89.7^{ab}	13.3^{c}
E100	98.4^{fg}	4.92^{ab}	4.78^{cd}	6.63	33.1^{c}	81.3^{de}	58.6^{b}
P100	115^{a}	5.34^{a}	6.18^{a}	6.60	36.2^{a}	83.6^{cde}	75.0^{a}
G50-E50	95.4^{g}	1.58^{de}	1.49^{ef}	6.61	31.0^{d}	84.1^{bcde}	17.7^{c}
G50-P50	101^{ef}	1.49^{e}	1.50^{ef}	6.60	33.4^{c}	78.7^{e}	19.2^{c}
E50-P50	107^{cd}	4.91^{ab}	5.27^{bc}	6.64	34.5^{bc}	86.1^{abcd}	61.1^{b}
G75-E12-P12	104^{de}	1.40^{e}	1.43^{ef}	6.58	30.8^{d}	88.5^{abc}	16.0^{c}
E75-G12-P12	103^{def}	4.24^{c}	4.29^{d}	6.61	33.8^{bc}	81.7^{de}	52.5^{b}
P75-G12-P12	114^{ab}	4.70^{bc}	5.31^{bc}	6.60	35.2^{ab}	91.3^{a}	58.6^{b}
G33-E33-P33	99.2^{efg}	2.08^{d}	2.07^{ef}	6.65	33.1^{c}	85.2^{bcd}	24.2^{c}
SEM	1.649	0.197	0.287	0.020	0.560	2.040	4.04
P-Value	<0.001	<0.001	<0.001	0.140	<0.001	0.002	<0.001

SEM: Standard error of the mean; ***, P<0.001; **, P<0.01; *, P<0.05; T, P<0.1; NS, P>0.1

Table 12: Fatty acids profile after 1-day in vitro incubation of Garlic, Eugenol, Polyphenol and their combinations

(%)	Acetate	Propionate	Butyrate	Valerate	BCFAs	A:P
CTL	63.9^{b}	20.2^{e}	9.78^{f}	2.00^{abc}	4.14^{ab}	3.19^{b}
G100	56.1^{e}	26.6^{a}	12.3^{cd}	1.76^{bc}	3.29^{c}	2.12^{e}
E100	65.7^{a}	17.5^{f}	11.4^{e}	1.66^{c}	3.75^{bc}	3.81^{a}
P100	63.9^{ab}	20.3^{e}	10.1^{f}	1.71^{c}	3.99^{b}	3.20^{b}
G50-E50	56.9^{e}	23.6^{c}	13.8^{a}	1.86^{bc}	3.79^{b}	2.44^{d}
G50-P50	53.6^{f}	26.6^{a}	13.4^{ab}	2.09^{abc}	4.20^{ab}	2.03^{e}
E50-P50	62.4^{bc}	20.4^{de}	11.5^{de}	1.80^{bc}	3.79^{b}	3.08^{b}
G75-E12-P12	56.1^{e}	25.2^{ab}	12.5^{c}	2.42^{a}	3.79^{b}	2.25^{de}
E75-G12-P12	59.9^{d}	21.8^{d}	12.4^{c}	1.84^{bc}	3.93^{b}	2.77^{c}
P75-G12-P12	61.5^{cd}	20.5^{de}	11.2^{e}	2.27^{ab}	4.55^{a}	3.05^{bc}
G33-E33-P33	56.7^{e}	24.4^{bc}	12.9^{bc}	2.09^{abc}	3.74^{bc}	2.33^{de}
SEM	0.616	0.513	0.286	0.184	0.165	0.106
P-Value	<0.001	<0.001	<0.001	0.122	0.002	<0.001

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; ***, P<0.001; **, P<0.01; *, P<0.05; T, P<0.1; NS, P>0.1

- ***Long term effect on CH_4 production and fermentation characteristics***

The long-term effects of the additives after 5 days of sequential incubations amplified the short-term effects described after one day of incubation (Table 13 and 14). Pure garlic (G100) reproduced the same scheme as described by day1. G100 promoted asignificantly increase in GP (+14 %), reduced NH_3-N (-12 %) and CH_4 (-69 %) (All P < 0.001), while maintaining higher VFA production than control (CTL) leading to a lower CH_4: VFA ratio. This was accompanied by a shifting in the fermentation from acetate (-18 %) towards butyrate (+77 %). Moreover, highproportions of valerate were observed on day 5 in almost all garlic-based combinations. Eugenol alone (E100) by day 5 reduced CH_4 emissions by 60 % and NH_3-N by 23 % (both P < 0.001), but this came at the cost of a 28 % decrease in total GP and a 24% decreaseof total VFAs (all P < 0.001) characterized by a decrease in the propionate molar proportion (-49 %) and elevated butyrate (+120%).In contrast, Polyphenol (P100) did not differ significantly from CTL for any parameter when studied after 5 days of incubation, with a markedly increased propionate by 14% while preserving acetate, and acetate-to-propionate ratio similar to that of the control.

Binary blends(G50–E50) and (G50–P50), as described by day 1, highlighted garlic antimethanogenic activity, reducing total CH_4 production by 42 % and 53 %, respectively (P < 0.001), while overall GP remained similar to the CTL with mirrored garlic's propionigenic and butyrogenic effects observed previously.Whereas (E50–P50)resembled the profile of Eugenol alone. Ternary mixtures exhibited a clear garlic-dose response: (G75–E12–P12) achieved maximal CH_4 suppression (-64%), and G33–E33–P33 intermediate reduction (-30%), whereas blends richer in Eugenol or Polyphenol (E75–G12–P12, P75–G12–E12) showed attenuated effects. These results further confirm that garlic is the primary driver of CH_4 inhibition, achieving significant CH_4 reductions without disrupting overall fermentation, being this antimethanogenic effect maintained over time. Ternary combinations exhibited a clear garlic-dose

dependency; (G75–E12–P12) suppressed acetate by 20% and enhanced butyrate by 70% whereas, (E75–G12–P12) resulted in a 59% reduction in propionate. Polyphenol maintained the control VFA pattern across most fatty acids. Overall, garlic maintained the same Day 1 pattern of shifting fermentation toward butyrate; as well as an increase in the valerate.

Table 13: Overall fermentation parameters after five days of *in vitro* incubation with Garlic, Eugenol and Polyphenol and their combinations

	Total GP (ml)	CH_4 (%)	Total CH_4 (ml)	pH	NH_3-N (mg/dl)	VFAs (mmol/L)	CH_4:VFAs
CTL	97.2 ^{bc}	3.78^{a}	3.90 ^{a}	6.59^{b}	23.8^{a}	58.2^{ab}	62.5^{ab}
G100	111 ^{a}	1.13^{e}	1.19 ^{d}	6.52^{d}	20.9^{bcd}	60.0^{abc}	20.0^{d}
E100	69.4^{d}	2.17^{cde}	1.49^{d}	6.66^{a}	18.3^{de}	44.0^{de}	35.3^{cd}
P100	102^{b}	3.62^{a}	3.98^{a}	6.61^{ab}	23.5^{ab}	56.1^{bc}	71.0^{a}
G50-E50	92.5^{c}	2.42^{bcd}	2.27^{cd}	6.59^{b}	17.6^{e}	51.6^{cd}	47^{bc}
G50-P50	98.2^{bc}	1.77^{cde}	1.74^{cd}	6.54^{cd}	21.5^{bc}	57.2^{bc}	31.4^{cd}
E50-P50	96.8^{bc}	3.58^{a}	3.71^{ab}	6.63^{ab}	21.8^{abc}	55.1^{bcd}	67.1^{a}
G75-E12-P12	101^{b}	1.43^{de}	1.39^{d}	6.52^{d}	19.7^{cde}	58.1^{abc}	24.4^{d}
E75-G12-P12	76.1^{d}	1.95^{cde}	1.52^{d}	6.66^{a}	17.3^{e}	40.1^{e}	38.3^{cd}
P75-G12- E12	96.3^{bc}	3.31^{ab}	3.51^{ab}	6.59^{b}	21.6^{bc}	59.1^{abc}	59.9^{ab}
G33-E33-P33	103^{b}	2.51^{e}	2.72^{bc}	6.59^{bc}	18.4^{de}	69.3^{a}	39.5^{cd}
SEM	2.406	0.367	0.411	0.018	1.057	3.910	6.8
P-Value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; ***. P<0.001; **. P<0.01; *. P<0.05; T. P<0.1; NS. P>0.1

Table 14: Fatty acids profile after five days of *in vitro* incubation with Garlic, Eugenol and Polyphenol and their combinations

(%)	Acetate	Propionate	Butyrate	Valerate	BCFA	A/P
CTL	57.4^{ab}	28.4^{a}	11.3^{e}	1.15^{c}	1.77^{a}	8.05^{c}
G100	47.0^{cde}	24.8^{bc}	20.1^{bc}	6.77^{a}	1.24^{abc}	1.91^{c}
E100	57.6^{a}	14.5^{d}	24.9^{abc}	1.22^{c}	1.77^{ab}	5.51^{a}
P100	52.7^{abc}	32.5^{a}	11.8^{de}	1.09^{c}	1.89^{a}	1.65^{c}
G50-E50	51.4^{bcd}	21.5^{c}	22.7^{bc}	3.22^{b}	1.12^{bc}	2.40^{bc}
G50-P50	44.7^{e}	29.3^{ab}	18.3^{cd}	6.16^{a}	1.51^{ab}	1.54^{c}
E50-P50	54.8^{ab}	14.7^{d}	26.5^{ab}	2.39^{bc}	1.67^{ab}	3.76^{b}
G75-E12-P12	46.0^{de}	27.1^{abc}	19.3^{bc}	5.83^{a}	1.75^{ab}	1.70^{c}
E75-G12-P12	55.2^{ab}	11.6^{d}	30.1^{a}	2.27^{bc}	0.782^{c}	5.41^{a}
P75-G12-E12	51.5^{bcd}	27.2^{abc}	17.7^{cd}	1.95^{bc}	1.67^{ab}	1.90^{c}
G33-E33-P33	53.9^{ab}	23.5^{bc}	18.9^{cd}	2.53^{bc}	1.09^{bc}	2.31^{bc}
SEM	2.037	2.272	2.509	0.604	0.242	0.554
P-Value	<0.001	<0.001	<0.001	<0.001	0.034	<0.001

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; ***. P<0.001; **. P<0.01; *. P<0.05; T. P<0.1; NS. P>0.1

- ***Comparison between Day 1 and Day 5 effects of Garlic, Eugenol, Polyphenol and their combinations***

A comparison of rumen *in vitro* fermentation parameters for all treatments on day 1 versus day 5 (Figure 13) shows a clear decline in overall fermentative activity over time: total GPdecreases by approximately 2–29%, and total VFA concentration falls by 15 to 50 %, inoculums and NH_3-N concentration drops by 29–49%. In contrast, ruminal pH remainedessentiallyconstant, indicating that the fermentation medium buffering capacity waspreserved. On day 1, garlic significantly inhibited CH_4 and NH_3-N and by day 5, garlic maintained its strong antimethanogenic and anti-proteolytic effects, with CH_4 reduction changing only slightly from (-78% to -69%) and NH_3-N reduction from (-16% to -12%). In contrast, eugenol's efficacy increased markedly over time: CH_4 inhibition deepened from -15% on day 1 to -61% on day 5, and NH_3-N reduction rose from -6% to -23%. Notably, the rank order of additive efficacy established on day 1 remains consistent through day 5 for garlic-based treatments. Although eugenol wasinitially less effective than garlic, it exhibits a delayed but substantial antimethanogenic and anti-proteolytic activity by day 5. Polyphenol, by contrast, remained inactive throughout the incubation and elicited the most pronounced VFA shift, showing the largest propionate molar proprortion by day 5. Garlic and eugenol treatments produced moderate butyrate enrichment with minimal acetate decreases. Overall, prolonged incubation amplified the transition from acetogenesis toward propionigenesis and butyrogenesis, with additive efficacy following the order: Polyphenol > Garlic > Eugenol (Figure 14).

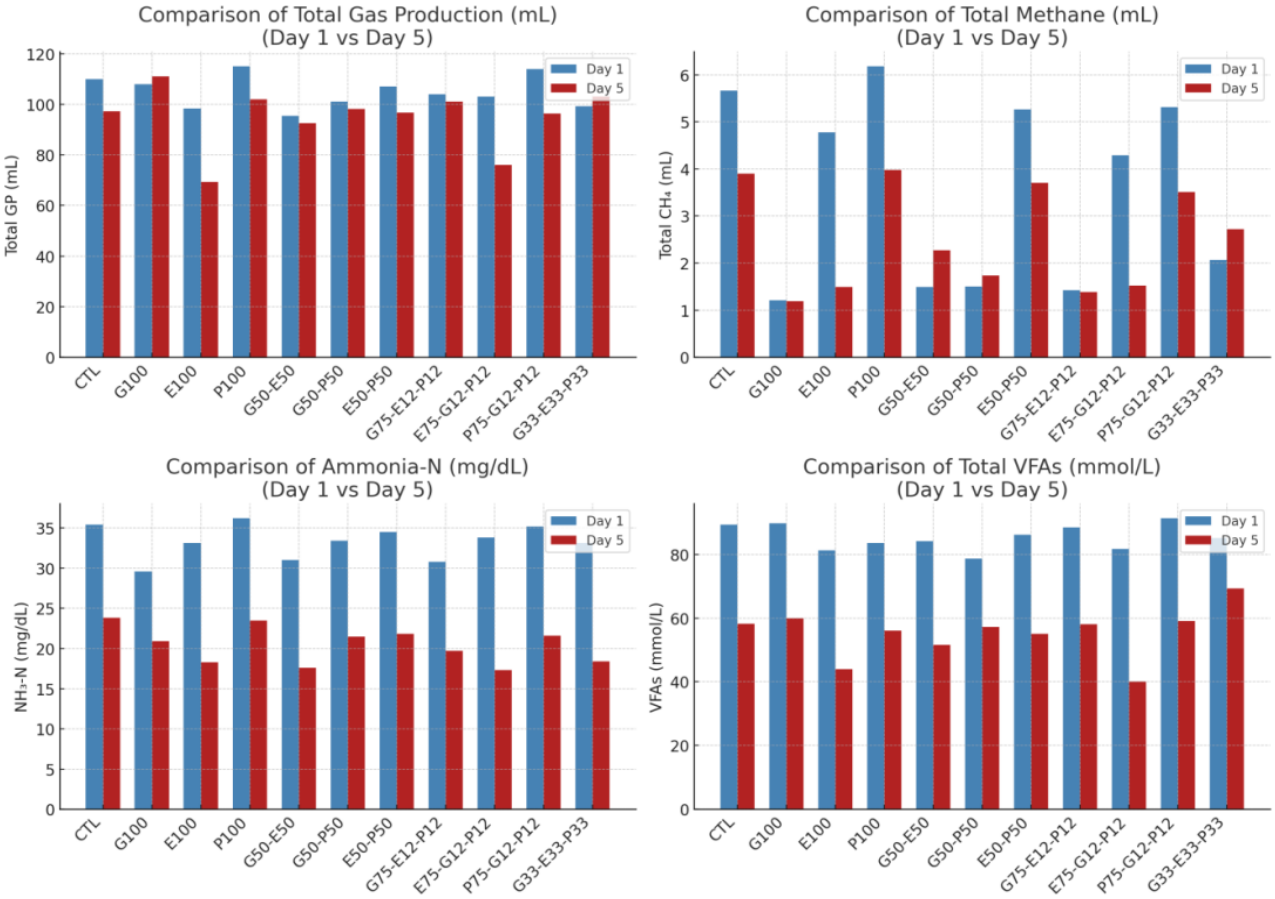


Figure 13*:* Fermentation parameters comparison between Day1 and Day5 *in vitro* incubation with Garlic, Eugenol, Polyphenol, and their combinations.

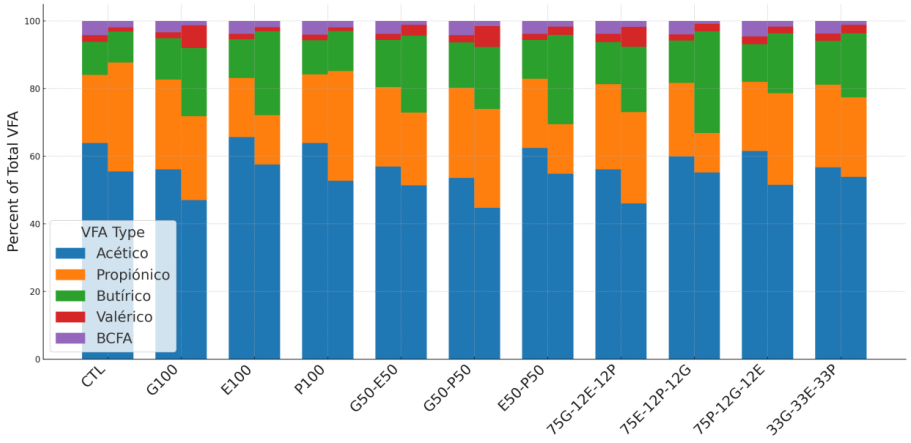


Figure 14*:* Fatty acids profil comparison between Day1 and Day5 *in vitro* incubation with Garlic, Eugenol, Polyphenol, and their combinations.

5.3 Experiment 3: *In vitro* Batch Incubations with higher doses

In this experiment, we tested different additives and, in some cases, higher dosages of main active compound tested in Experiment 1 due to the inconclusive otcomes. Garlic1, wasa garlic extract containing 5.57% of diallyl disulphide as the main active compound. Supplementation with increasing doses of it had no effect on GP ($p<0.05$) while reduced CH_4 concentration linearly (-56%, $p<0.01$). As a result, Garlic 1 led to a substantial linear decrease in the total CH_4 emissions. In relation with the rumen fermentation pattern, Garlic1promoted a linear decrease in the pH and tended to linearly decrease NH_3-N concentration. Supplementation with Garlic1 did not modify the total VFAs concentration nor the proportion of the main VFAs but linearly decreased the BCFA (-38%,$p<0.01$) and the CH_4/VFA ratio. These findings suggest that Garlic1 was a promising additive to decrease CH_4 emissions without inhibiting feed degradation or promoting alteration of the rumen fermentation pattern (Table 15).

Garlic 2 was a different garlic extract containing 4.87% of diallyldisulphide as the main active compound. Increasing doses ofGarlic 2 tended to increase the total GP and decrease CH_4 in the gas. This resulted in a lower total CH_4 production (up to -24%, $P<0.05$) being lower than reported for Garlic1. Rumen pH and concentrations of total NH_3-N were not affected by dietary supplementation with Garlic2. However, this additive when supplemented at the highest dose led to profound shift in the fermentation pattern. In fact, the highest dose of Garlic2 promoted a decrease in the total VFA concentration indicating a negative effect in the rumen function. However, moderate doses of this additive promoted a decrease in the butyrate and BCFA molar proportions as well as an increase in the A/P ratio. These findings suggest that moderate doses of Garlic2 can potentially favour the feed fermentation in terms of higher GP and A/P ratio. However higher doses can inhibit to some extent the rumen fermentation

Table 15: Effect of increasing doses of Garlic1 and Garlic2 used in experiment 3 on *in vitro* gas production and fermentation characteristics.

	Active compound dose (mg/gDM)							
Garlic1	**0**	**1.5**	**3**	**6**	**12**	SEM	P-value	Contrast
pH	6.58	6.58	6.57	6.57	6.56	0.006	T	L^{**}
NH3-N(mg/dL)	29.5	28.9	28.4	25.8	23.5	2.370	NS	L^{T}
Total VFA (mmol/L)	60.4	58.4	54.0	68.2	63.0	4.430	NS	NS
Acetate (%)	56.0	54.3	49.9	58.3	55.6	2.134	NS	NS
Propionate (%)	25.9	27.1	29.7	26.6	27.8	1.290	NS	NS
Butyrate (%)	11.6	11.9	13.1	10.3	12.1	0.703	NS	NS
Valerate(%)	1.90^{a}	2.04^{ab}	2.30^{b}	1.72^{a}	1.84^{a}	0.114	*	NS
BCFA.(%)	6.46^{b}	6.35^{b}	7.14^{b}	4.23^{a}	3.97^{a}	0.592	**	L^{**}
A/P Ratio	2.18	2.05	1.69	2.38	2.17	0.234	NS	NS
Total GP (mL)	99.4	101.0	98.6	98.0	98.9	1.889	NS	NS
CH_4 (%)	4.35^{a}	4.43^{b}	3.73^{bc}	3.11^{c}	1.91^{c}	0.315	***	L^{***}
Total CH_4 (mL)	4.51^{c}	4.56^{c}	3.80^{bc}	3.16^{b}	1.93^{a}	0.313	***	L^{***}
CH_4/VFA (mL/mol)	77.0^{b}	78.7^{b}	79.1^{b}	49.2^{a}	34.1^{a}	6.23	***	L^{***}
Garlic2								
pH	6.58	6.58	6.59	6.58	6.60	0.008	NS	L^{T}
NH3-N(mg/dL)	29.5	27.7	28.4	28.2	28.2	2.084	NS	NS
Total VFA (mmol/L)	60.4^{b}	64.2^{b}	63.2^{b}	63.6^{b}	51.0^{a}	2.090	**	$L^{**}.Q^{**}$
Acetate (%)	56.0^{b}	60.3^{b}	60.2^{b}	61.5^{b}	53.2^{a}	1.274	**	$L^{*}.Q^{***}$
Propionate (%)	25.9^{b}	23.7^{a}	23.6^{a}	23.2^{a}	26.6^{b}	0.707	*	Q^{**}
Butyrate (%)	11.6^{b}	10.4^{a}	10.5^{a}	10.1^{a}	12.8^{c}	0.365	***	$L^{**}.Q^{***}$
Valerate(%)	1.90^{b}	1.61^{a}	1.62^{ab}	1.59^{a}	2.33^{c}	0.092	***	$L^{***}.Q^{***}$
BCFA.(%)	6.46^{bc}	5.44^{ab}	5.44^{ab}	5.01^{a}	6.83^{c}	0.385	*	Q^{**}
A/P Ratio	2.18^{a}	2.58^{b}	2.60^{b}	2.75^{b}	2.06^{a}	0.125	**	Q^{***}
Total GP (mL)	99.4^{a}	103^{abc}	104^{c}	104^{c}	101^{ab}	1.183	*	Q^{**}
CH4 (%)	7.09^{b}	6.93^{b}	6.87^{b}	6.81^{b}	5.60^{a}	0.320	*	L^{**}
Total CH4 (mL)	4.51^{b}	4.29^{b}	4.28^{b}	4.25^{b}	3.41^{a}	0.181	**	L^{***}
CH4/VFA (mL/mol)	77.0	69.0	69.5	68.9	65.4	4.110	NS	NS

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; L. linear response; Q. quadratic response. ***. P<0.001; **. P<0.01; *. P<0.05; T. P<0.1; NS. P>0.1

MIX was made as a mixture of 70% Polyphenol10, 20% of Eugenol, and 10% of Garlic1. Supplementation with MIX promoted a linear and relevant decrease in total CH_4 production (up to -47%). Moreover, supplementation with MIX promoted a minor inhibition of the rumen fermentation as no differences were noted in terms of total VFA concentration and only the higher dose (88mg/gDM) led to a moderate decrease in the total GP (-10%). In addition, supplementation with MIX had minor effects in the rumen fermentation and only tended to promote a linear decrease in the pH and NH_3-N. These results suggest that supplementation with MIX can lead to a significant reduction of CH_4 production without impairing or inhibiting the rumen fermentation as described in (Table 16). These results were similar to those obtained with Garlic1 indicating that diallyldisulfide may be the main active compound in MIX.

Table16: Effect of increasing doses of MIX on *in vitro* gas production and fermentation characteristics.

	Dose. mg/gDM							
MIX	0	11	22	44	88	SEM	P-value	Contrast
pH	6.58	6.56	6.56	6.55	6.55	0.007	T	$L^{T}.Q^{T}$
NH3-N(mg/dL)	29.5	26.7	26.5	23.6	20.8	1.980	T	L^{**}
Total VFA (mmol/L)	60.4	58.5	65.9	56.4	55.6	3.460	NS	NS
Acetate (%)	56.0	60.0	61.4	60.3	59.4	1.599	NS	NS
Propionate (%)	25.9	24.0	23.7	24.2	25.4	0.902	NS	NS
Butyrate (%)	11.6	10.3	9.70	10.2	10.4	0.405	T	L^{*}
Valerate(%)	1.90^{b}	1.58^{a}	1.42^{a}	1.41^{a}	1.40^{a}	0.073	***	$L^{**}.Q^{**}$
BCFA.(%)	6.46	5.33	5.00	5.10	4.90	0.460	NS	L^{T}
A/P Ratio	2.18	2.60	2.67	2.54	2.36	0.177	NS	NS
Total GP (mL)	99.4^{b}	101^{b}	102^{b}	99.6^{b}	89.6^{a}	1.398	***	$L^{***}.Q^{**}$
CH_4 (%)	4.35^{d}	3.32^{c}	3.36^{c}	2.93^{b}	2.30^{a}	0.046	***	$L^{***}.Q^{***}$
Total CH_4 (mL)	4.51^{d}	4.04^{c}	4.16^{c}	3.58^{b}	2.61^{a}	0.088	***	L^{***}
CH_4/VFA (mL/mol)	77.0^{c}	70.4^{bc}	66.1^{b}	65.9^{b}	51.4^{a}	3.470	**	L^{***}

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; L. linear response; Q. quadratic response.
***. P<0.001; **. P<0.01; *. P<0.05; T. P<0.1; NS. P>0.1

Polyphenol6 is a polyphenol source containing Gallic acid as the main active compound. Increasing doses of this polyphenol promoted a linear increase in total GP (+23%). In terms of CH_4 production a quadratic effect was detected as the lowest percentage of CH_4 and the lowest CH_4 production was observed with intermediate doses of this compound (-21% at 3 mg/gDM). Whereas an increase was noted with the highest dose (+49% at 3 mg/gDM). Increasing supplementation with Polyphenol6 did not modify the rumen fermentation in terms of NH_3-N and total VFA concentration but the highest dose promoted a decrease in the pH. Interestingly, supplementation with Polyphenol6 led to a shift in the fermentation pattern characterized by an increase in the acetate and a decrease in the rest of individual VFA; being this effect observed for all levels of supplementation. These findings suggest that Polyphenol6 may have a microbial modulatory activity favouring the activity of fibrolytic microbes and ultimately the A/P ratio. Moreover, the use of Polyphenol6 at moderate doses could have certain capacity to inhibit CH_4 production without compromising the VFA production. Polyphenol12 wasanother source of gallic acid which showed no effects in the GP or rumen fermentation when used even at high doses (up to 200mg/g DM) as described (Table 17).

Table 17: Effects of increasing doses of Polyphenols 6 and 12 used in experiment 3 *in vitro* gas production and fermentation characteristics

	Active compound dose (mg/gDM)					SEM	P-value	Contrasts
Polyphenol 6	**0**	**1.5**	**3**	**6**	**12**			
pH	6.58^{b}	6.61^{b}	6.61^{b}	6.58^{b}	6.42^{a}	0.015	***	L^{***}. Q^{***}
NH3-N(mg/dL)	29.5	27.0	28.3	28.9	30.1	2.350	NS	NS
Total VFA (mmol/L)	60.4	77.3	73.1	78.3	81.4	6.830	NS	NS
Acetate (%)	56.0^{a}	66.3^{b}	64.6^{b}	67.3^{b}	65.0^{b}	1.797	**	$L^{*}.Q^{**}$
Propionate (%)	25.9^{b}	21.0^{a}	21.1^{a}	19.8^{a}	21.1^{a}	1.019	*	$L^{*}.Q^{*}$
Butyrate (%)	11.6^{b}	8.38^{a}	9.22^{a}	8.33^{a}	9.02^{a}	0.532	**	$L^{*}.Q^{**}$
Valerate(%)	1.90^{b}	1.27^{a}	1.48^{a}	1.35^{a}	1.51^{a}	0.099	**	Q^{**}
BCFA.(%)	6.46^{b}	4.42^{a}	4.94^{a}	4.49^{a}	4.77^{a}	0.343	**	$L^{*}.Q^{*}$
A/P Ratio	2.18^{a}	3.17^{b}	3.08^{b}	3.43^{b}	3.15^{b}	0.229	*	$L^{*}.Q^{*}$
Total GP (mL)	99.4^{a}	120^{cd}	107^{b}	116^{c}	122^{d}	1.799	***	$L^{***}.Q^{T}$
CH_4 (%)	4.35^{b}	3.93^{ab}	3.42^{a}	4.60^{bc}	5.88^{c}	0.232	***	$L^{***}.Q^{**}$
Total CH_4 (mL)	4.51^{bc}	4.28^{ab}	3.54^{a}	5.19^{c}	6.73^{d}	0.250	***	$L^{***}.Q^{*}$
CH_4/VFA (mL/mol)	77.0	71.0	71.0	67.0	74.7	6.250	NS	NS
Polyphenol 12	**0**	**0.15**	**0.3**	**0.6**	**1.2**			
pH	6.58	6.57	6.57	6.57	6.57	0.004	NS	NS
NH3-N(mg/dL)	29.5	27.8	28.3	28.6	28.6	1.933	NS	NS
Total VFA (mmol/L)	60.4	59.8	59.3	56.3	57.9	4.410	NS	NS
Acetate (%)	56.0	61.3	60.0	58.8	59.3	2.066	NS	NS
Propionate (%)	25.9	23.0	23.6	23.8	23.8	1.009	NS	NS
Butyrate (%)	11.6	10.0	10.4	10.7	10.6	0.616	NS	NS
Valerate(%)	60.4	59.8	59.3	56.3	57.9	4.410	NS	NS
BCFA.(%)	6.46	5.25	5.51	6.15	5.80	0.541	NS	NS
A/P Ratio	2.18	2.75	2.66	2.50	2.53	0.194	NS	NS
Total GP (mL)	99.4	100.3	99.4	97.5	99.8	1.258	NS	NS
CH_4 (%)	4.35	4.41	4.40	4.37	4.37	0.099	NS	L^{T}
Total CH_4 (mL)	4.51	4.61	4.56	4.45	4.52	0.097	NS	NS
CH_4/VFA (mL/mol)	77.0	79.3	81.5	81.1	73.6	5.460	NS	NS

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; L. linear response; Q. quadratic response. ***. P<0.001; **. P<0.01; *. P<0.05; T. P<0.1; NS. P>0.1

Polyphenol13 and Polyphenol14, both derived from Scutellariabaicalensis, moderately increased totalGPwithout significantly affecting CH_4 production, while inducing a linear decrease in rumen pH and shifting the fermentation profile toward acetate as described in (Table 18) mainly at the expense of propionate for Polyphenol13 and butyrate for Polyphenol14; these effects remained modest due to the relatively low doses used in the experiment.

Table 18: Effects of increasing doses of Polyphenols 13 and 14 used in experiment 3 in vitro gas production and fermentation characteristics

	Active compound dose (mg/gDM)							
	0	**0.15**	**0.3**	**0.6**	**1.2**	SEM	P-value	Contrasts
Polyphenol 13								
pH	6.58^{c}	6.57^{c}	6.55^{a}	6.58^{c}	6.56^{b}	0.003	***	L^{*}
NH3-N(mg/dL)	29.5	27.9	28.4	28.1	28.5	1.927	NS	NS
Total VFA (mmol/L)	60.4	71.5	70.0	61.1	71.6	5.380	NS	NS
Acetate (%)	56.0	63.9	62.6	60.9	60.7	1.746	T	Q^{T}
Propionate (%)	25.9^{b}	21.6^{a}	22.0^{a}	22.5^{a}	23.1^{ab}	0.937	*	Q^{*}
Butyrate (%)	11.6	9.29	9.61	10.5	10.4	0.532	T	NS
Valerate(%)	1.90	1.61	1.76	1.78	1.68	0.111	NS	NS
BCFA.(%)	6.46	5.38	5.83	6.11	6.05	0.316	NS	NS
A/P Ratio	2.18	2.96	2.86	2.74	2.66	0.171	T	Q^{T}
Total GP (mL)	99.4	98.4	102	99.7	104	1.190	T	L^{*}
CH_4 (%)	4.35	4.53	4.07	4.40	4.48	0.108	T	NS
Total CH_4 (mL)	4.51	4.59	4.30	4.53	4.80	0.126	NS	L^{T}
CH_4/VFA (mL/mol)	77.0	69.9	67.0	76.6	72.7	4.630	NS	NS
Polyphenol 14								
pH	6.58^{b}	6.56^{a}	6.57^{ab}	6.57^{ab}	6.56^{a}	0.003	*	L^{T}
NH3-N(mg/dL)	29.5	28.9	28.5	28.6	28.7	1.927	NS	NS
Total VFA (mmol/L)	60.4	67.2	73.9	63.2	61.2	5.020	NS	NS
Acetate (%)	56.0	60.0	65.2	63.1	62.2	2.620	NS	Q^{T}
Propionate (%)	25.9	23.3	20.9	21.9	22.4	1.286	NS	Q^{T}
Butyrate (%)	11.6	10.5	8.82	9.60	9.82	0.799	NS	Q^{T}
Valerate(%)	1.90	1.72	1.41	1.59	1.60	0.149	NS	NS
BCFA.(%)	6.46	6.27	4.83	4.95	5.12	0.761	NS	NS
A/P Ratio	2.18	2.60	3.15	3.01	2.89	0.292	NS	Q^{T}
Total GP (mL)	99.4^{a}	104^{c}	104^{c}	101^{ab}	102^{abc}	1.010	*	Q^{T}
CH_4 (%)	4.35	4.43	4.59	4.37	4.35	0.131	NS	NS
Total CH_4 (mL)	4.51	4.73	4.91	4.58	4.58	0.165	NS	NS
CH_4/VFA (mL/mol)	77.0	76.7	71.6	75.6	77.2	5.960	NS	NS

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; L. linear response; Q. quadratic response. ***. P<0.001; **. P<0.01; *. P<0.05; T. P<0.1; NS. P>0.1

Dietary supplementation with cinnamonpromoted deep changes in the*in vitro* rumen fermentation. Increasing doses of cinnamon led to a linear decrease in the total GP leading to a significant inhibition of the fermentation when used at high doses (100-200 mg/gDM) in terms of GP and total VFA production indicating an excessive anti-microbial activity. This inhibition of the GP was also accompanied by a decrease in the CH_4 emissions in terms of percentage of gas and ml of CH_4 produced (up to -98 %, $P < 0.001$). Interestingly the use of Cinnamon at low doses (25 and 50mg/gDM) did not promote changes in terms of GP, CH_4 production, pH, NH_3-N, total VFAs and individual VFA concentrations suggesting the absence of negative effects for the rumen fermentation. These findings suggest that Cinnamon has a very narrow range of concentrations to improve rumen function as at low doses does not promote differences with the control whereas at high doses inhibits the rumen fermentation to a great extent (Table19).

Oregano essential oil is rich in Carvacrol as the main bioactive compound. Increasing doses of Oregano promoted a partial inhibition of the rumen fermentation as noted by a linear decrease in the GP, CH_4production and total VFA and NH_3-N concentrations, as well as an increase in the rumen pH. The lowest dose did not change any of the parameters analyzed. Interestingly the second dose (50 mg/g DM) promoted a moderate decrease in the total GP (32%) which was accompanied by a decrease in CH_4 production (-45%) and a smaller decrease in the total VFA concentration (-14%) without affecting the proportions of individual VFA indicating that this dose may be close to the optimal to improve the rumen function. Higher doses (100 and 200mg/gDM) promoted a nearly complete inhibition of CH_4 production (-96%) but also had a negative impact in the feed digestion as noted by a strong inhibition of the GP (-79 and -83%) and total VFA concentration (-55% and -63%) without affecting the proportions of individual VFA. These results suggest that Oregano has an ability to inhibit rumen methanogenesis but negative effects in the feed fermentation can occur when used at high doses (Table19)

Table 19: Effects of increasing doses of Cinnamon and Oregano used in experiment 3 on *in vitro*gas production and fermentation characteristics.

	Active compound dose (mg/gDM)							
	0	**25**	**50**	**100**	**200**	SEM	P-value	Contrasts
Cinnamon								
pH	6.58^{a}	6.57^{a}	6.58^{a}	6.61^{a}	6.72^{b}	0.022	**	L^{***}
NH3-N(mg/dL)	29.5^{c}	27.3^{bc}	23.6^{abc}	23.0^{ab}	19.8^{a}	1.939	*	L^{**}
Total VFA (mmol/L)	60.4^{b}	56.5^{b}	51.5^{b}	33.6^{a}	23.0^{a}	4.30	***	L^{***}
Acetate (%)	56.0^{a}	58.7^{a}	59.0^{a}	59.6^{a}	68.7^{b}	2.60	*	L^{**}
Propionate (%)	25.9^{b}	24.1^{b}	23.6^{b}	15.9^{a}	16.0^{a}	1.290	***	$L^{***}.Q^{*}$
Butyrate (%)	11.6^{a}	11.1^{a}	11.7^{a}	18.1^{b}	9.67^{a}	1.319	**	Q^{**}
Valerate(%)	1.90^{a}	1.82^{a}	1.89^{a}	2.59^{b}	1.43^{a}	0.222	*	Q^{*}
BCFA.(%)	6.46	5.66	5.40	6.89	6.81	0.729	NS	NS
A/P Ratio	2.18^{a}	2.55^{a}	2.55^{a}	3.89^{b}	4.52^{b}	0.382	**	L^{***}
Total GP (mL)	99.4^{c}	98.3^{c}	94.8^{c}	59.8^{b}	29.6^{a}	3.82	***	L^{***}
CH_4 (%)	4.35^{c}	4.43^{c}	3.81^{c}	1.30^{b}	0.260^{a}	0.257	***	$L^{***}.Q^{T}$
Total CH_4 (mL)	4.51^{c}	4.63^{c}	3.93^{c}	1.09^{b}	0.102^{a}	0.344	***	$L^{***}.Q^{T}$
CH_4/VFA (mL/mol)	77.0^{c}	84.7^{c}	76.4^{c}	29.2^{b}	4.22^{a}	7.26	***	L^{***}
Oregano								
pH	6.58^{a}	6.59^{a}	6.66^{bc}	6.74^{c}	6.77^{d}	0.007	***	$L^{***}.Q^{***}$
NH3-N(mg/dL)	29.5^{b}	27.0^{ab}	23.3^{ab}	20.7^{a}	20.5^{a}	2.230	*	$L^{*}.Q^{T}$
Total VFA (mmol/L)	60.4^{b}	51.1^{b}	51.9^{b}	27.5^{a}	22.1^{a}	3.300	***	$L^{***}.Q^{*}$
Acetate (%)	56.0	55.8	59.6	60.3	66.1	3.080	NS	L^{*}
Propionate (%)	25.9	24.5	18.3	17.5	18.2	2.580	NS	$L^{*}.Q^{T}$
Butyrate (%)	11.6	13.3	16.3	15.8	9.37	1.726	T	Q^{*}
Valerate(%)	1.90	2.04	1.86	1.96	1.39	0.190	NS	L^{*}
BCFA.(%)	6.46	6.56	8.03	8.42	7.44	1.297	NS	NS
A/P Ratio	2.18	2.33	3.91	3.52	4.07	0.697	NS	L^{T}
Total GP (mL)	99.4^{c}	99.2^{c}	67.4^{b}	20.7^{a}	17.3^{a}	3.000	***	$L^{***}.Q^{***}$
CH_4 (%)	4.35^{c}	4.27^{c}	3.26^{b}	0.910^{a}	0.713^{a}	0.247	***	$L^{***}.Q^{***}$
Total CH_4 (mL)	4.51^{c}	4.39^{c}	2.48^{b}	0.192^{a}	0.160^{a}	0.339	***	$L^{***}.Q^{***}$
CH_4/VFA (mL/mol)	77.0^{c}	87.2^{c}	47.0^{b}	6.57^{a}	5.78^{a}	7.230	***	$L^{***}.Q^{**}$

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; L. linear response; Q. quadratic response. ***. P<0.001; **. P<0.01; *. P<0.05; T. P<0.1; NS. P>0.1

The positive control was sodium 2-bromoethanesulphonate (BES) which is a potent CH_4inhibitor. Results showed that supplementation with increasing doses of BES promoted a linear but small decrease in GP (up to -18%) which was not detected in terms of total VFA concentration but led to an important linear decrease in the CH_4 production (from -37% to -75%). Despite this intensive CH_4 inhibition, BES supplementation did not modify the fermentation pattern in terms of pH, NH_3-N, total VFA and the proportion of individual VFA. These results confirmed that BES is a potent antimethanogenic additive which valid to test hypothesis in vitro (Table20).

Table 20: Effects of increasing doses BES used in experiment 3 on *in vitro* gas production and fermentation characteristics

	Active compound dose (mg/gDM)							
	0	**0.05**	**0.1**	**0.2**	**0.4**	SEM	P-value	Contrasts
BES								
pH	6.58	6.59	6.60	6.61	6.65	0.021	NS	L^{*}
NH3-N(mg/dL)	29.5	26.7	28.2	25.9	24.9	2.006	NS	NS
Total VFA (mmol/L)	60.4	68.2	57.3	70.3	56.5	8.280	NS	NS
Acetate (%)	56.0	63.5	56.0	58.9	55.2	2.108	T	NS
Propionate (%)	25.9	23.0	26.1	24.9	27.4	0.956	T	L^{T}
Butyrate (%)	11.6	9.11	11.7	10.6	11.8	0.706	T	NS
Valerate(%)	1.90	1.38	1.98	1.82	1.88	0.167	NS	NS
BCFA.(%)	6.45	5.22	6.44	6.03	5.97	0.393	NS	NS
A/P Ratio	2.18	2.82	2.24	2.37	2.03	0.186	T	NS
Total GP (mL)	99.4^{c}	91.4^{b}	90.1^{b}	87.5^{b}	81.8^{b}	1.604	***	$L^{***}.Q^{T}$
CH_4 (%)	4.35^{c}	2.93^{b}	2.80^{b}	2.32^{ab}	1.38^{a}	0.310	***	$L^{***}.Q^{T}$
Total CH_4 (mL)	4.51^{c}	2.84^{b}	2.68^{b}	2.11^{ab}	1.15^{a}	0.363	***	$L^{***}.Q^{T}$
CH_4/VFA (mL/mol)	77.0^{c}	43.5^{b}	46.8^{b}	33.9^{ab}	22.5^{a}	6.150	***	$L^{***}.Q^{*}$

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; L. linear response; Q. quadratic response. ***. P<0.001; **. P<0.01; *. P<0.05; T. P<0.1; NS. P>0.1

5.4 Experiment 4: Short-and long-term incubation of additives combinations from Experiment 3

- **Gas production over 5 days *in vitro* incubation**

As shown in Figure 15, over the extended five-days of consecutive incubations, all treatments exhibited the characteristic Day 1 to Day 2 decrease in GP, followed by divergent recovery trajectories. The control (CTL) decreased by 20% by day 2 then only 12% by Day 5. Garlic (G100) maintained the most vigorous fermentation, dropping to (-20%) by day 2 before climbing to (+2%)by day 5, whereas cinnamon (C100) suffered the greatest inhibition plummeting by (-65%) with no subsequent recovery followed by Oregano (O100) dipping to (-55%) both with no subsequent recovery.Binary blends combined these effects: G50–C50 and G50–O50 followed garlic's resilience with slumps to (-40%) and full recoveries (-4%), whereas C50–O50 though initially inhibited (-66%), recovered better (-35%) than either cinnamon or oregano alone. Ternary mixtures again demonstrated a garlic-dose dependency: G75–C12–O12 remained above 80 mL throughout and peaked at 104 mL, G12–C75–O12 tracked cinnamon's deep Day 2 slump (-66%) with a better recovery at day 5 (-46%), G12-C12-O75 paralleled the Oregano trajectory. Lastly, the equal dose blend exhibited an intermediate trajectory, declining by 43% on day 2 before partially rebounding (-24%) by day 5. These dynamics emphasize garlic's ability to sustain and even enhance fermentative activity in the presence of potent inhibitors like cinnamon. In

conclusion, extending incubation to five days allows capture both immediate and delayed microbial responses and stability of each additive.

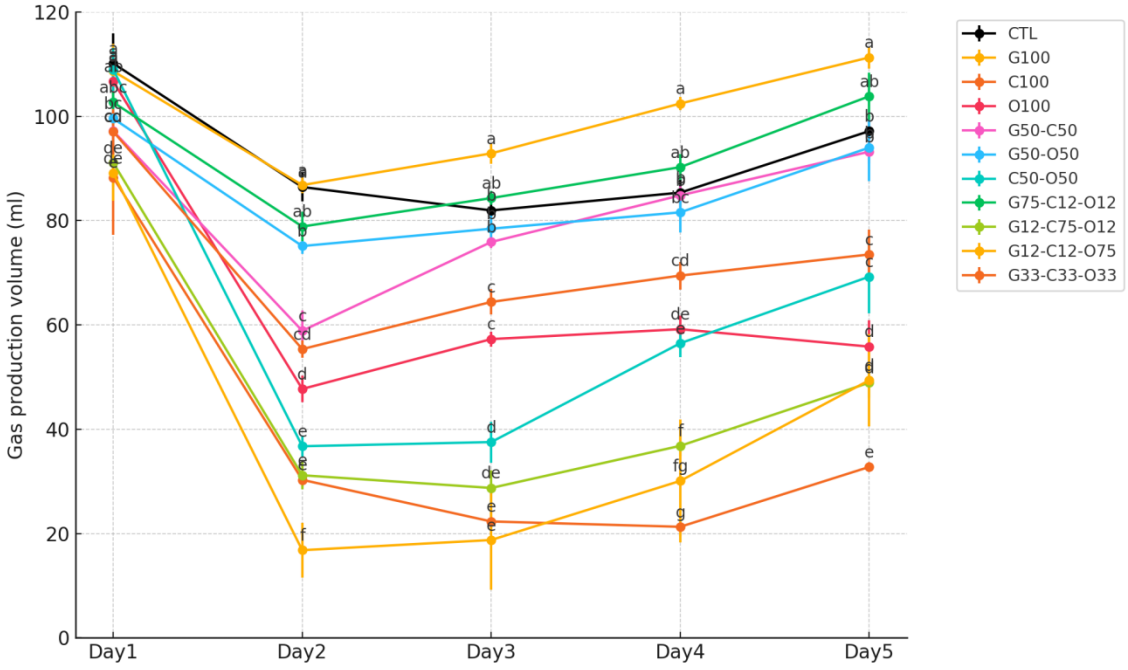


Figure 15: Gas production over 5 days in vitro incubation of Garlic, Cinnamon, Oregano and their combinations

- **Short term Fermentation**

In this incubation, garlic (G100) once again emerged as the most effective additive, reducing CH_4 by 78% compared to the control ($P < 0.001$), while maintaining high total GP, stable pH and VFA levels, and significantly lowering NH_3–N by 16%. Garlic also induced a marked shift in fermentation pattern, decreasing acetate by 12% while increasing propionate by 32% and butyrate by 26%, resulting in a collapse of the acetate: propionate ratio from 3.2 to 2.1. In comparison, cinnamon (C100) achieved a moderate CH_4 reduction (-45%) and lowered NH_3–N by 15%, but this came at the cost of total gas (-20%) and VFA production (-23%). Cinnamon altered the VFA profile by reducing propionate (-14%) and strongly increasing butyrate (+61%), with acetate remaining relatively stable. Adding Oregano (O100) showed no significant impact on GP or CH_4but reduced total VFAs by 14% and markedly altered the fermentation profile as the same way as Cinnamon by suppressing propionate (-22%) and enhancing butyrate (+52%). Binary blends (G50–C50) or oregano (G50–O50) maintained high CH_4 inhibition (>70%), while supporting moderate GP (~100 mL) and lowering NH_3–N similarly to garlic alone. These blends largely preserved garlic's propionigenic effect, with acetate reductions (-11 to -15%) and propionate increases (+20 to +30%). By contrast C50–O50 behaved similarly to the control, both in terms of CH_4 and fermentation profile. Ternary blends, (G75–C12–O12) achieved the highest CH_4 reduction (-73%), while preserving garlic's fermentation shift with +25% propionate and (-12%) acetate; (G33–C33–O33) resulted in intermediate CH_4 reduction (–56%), with similar VFA shifts (-15% acetate, +15% propionate);In contrast, garlic-poor blends like (G12–C75–O12) and (G12–C12–O75) showed attenuated CH_4 reduction (-57% and –34%), and followed the fermentation pattern of cinnamon and oregano, respectively. The cinnamon-rich blend mimicked C100, while the oregano-rich blend yielded the strongest butyrate increase (+90%) and deepest propionate suppression (-32%).

Together, these results confirm that garlic is the key additive for simultaneously reducing CH_4 and enhancing fermentation efficiency via propionate promotion, while cinnamon and oregano exert more selective effects, favoring butyrate formation and often inhibitingoverall fermentation when used alone or in excess (Tables 21–22).

Table 21: Overall fermentation parameters after 1-day in vitro incubation of Garlic, Cinnamon and Oregano and their combinations

	Total GP (ml)	CH_4 (%)	TotalCH_4 (ml)	pH	NH_3-N (mg/dl)	VFAs (mmol/L)	CH_4:VFAs
CTL	110^{a}	5.14^{a}	5.67^{a}	6.58^{c}	35.4^{a}	89.3^{a}	63.5^{ab}
G100	109^{a}	1.10^{e}	1.21^{e}	6.57^{c}	29.6^{bc}	89.7^{a}	13.4^{f}
C100	88^{e}	2.79^{c}	3.08^{bc}	6.67^{ab}	30.1^{bc}	68.8^{d}	44.8^{cd}
O100	107^{ab}	4.92^{a}	5.31^{a}	6.66^{ab}	32.0^{b}	76.4^{bcd}	69.5^{a}
G50-C50	97^{cd}	1.70^{de}	1.64^{de}	6.61^{bc}	29.8^{bc}	80.0^{abc}	20.5^{ef}
G50-O50	100^{bc}	1.47^{e}	1.47^{de}	6.62^{abc}	30.4^{bc}	81.6^{abc}	18.0^{ef}
C50-O50	109^{a}	5.05^{a}	5.57^{a}	6.58^{c}	31.7^{b}	83.8^{abc}	66.4^{ab}
G75-C12-O12	103^{abc}	1.44^{e}	1.48^{de}	6.64^{abc}	28.7^{c}	85.4^{ab}	17.3^{ef}
G12-C75-O12	91^{de}	2.41^{cd}	2.41^{cd}	6.68^{ab}	31.1^{bc}	74.1^{cd}	32.5^{de}
G12-C12-O75	89^{de}	3.95^{b}	3.72^{b}	6.69^{ab}	29.3^{bc}	74.5^{cd}	49.9^{bc}
G33-C33-O33	97^{cd}	2.57^{cd}	2.51^{cd}	6.65^{ab}	31.4^{bc}	77.4^{bcd}	32.4^{de}
SEM	1.649	0.197	0.287	0.020	0.560	2.040	4.04
P-Value	<0.001	<0.001	<0.001	0.140	<0.001	0.002	<0.001

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; ***. P<0.001; **. P<0.01; *. P<0.05; T. P<0.1; NS. P>0.1

Table 22: Fatty acids profile after 1 day *in vitro* incubation of Garlic, Cinnamon and Oregano and their combinations

(%)	Acetate	Propionate	Butyrate	Valerate	BCFAs	A:P
CTL	63.9^{a}	20.2^{cd}	9.80^{g}	2.00^{bcd}	4.14^{ab}	3.20^{cd}
G100	56.1^{cd}	26.6^{a}	12.3^{ef}	1.77^{d}	3.29^{de}	2.12^{e}
C100	61^{abc}	17.3^{de}	15.8^{bc}	2.43^{abc}	3.52^{cde}	3.58^{bc}
O100	63.5^{a}	15.8^{e}	14.8^{cd}	2.04^{bcd}	3.78^{abcd}	4.04^{ab}
G50-C50	56.9^{bcd}	24.1^{abc}	13.3^{de}	2.01^{bcd}	3.68^{abcde}	2.48^{de}
G50-O50	54.1^{d}	26.3^{a}	13.9^{cde}	2.12^{bcd}	3.59^{bcde}	2.08^{e}
C50-O50	62.8^{a}	21.2^{bcd}	10.5^{fg}	1.87^{cd}	3.67^{abcde}	2.98^{cd}
G75-C12-O12	55.9^{d}	25.2^{ab}	13.7^{cde}	1.98^{cd}	3.18^{e}	2.24^{e}
G12-C75-O12	55.6^{d}	20.7^{cd}	17.5^{ab}	2.47^{bcd}	$3.72a^{bcd}$	2.71^{de}
G12-C12-O75	61.2^{ab}	13.7^{e}	18.6^{a}	2.53^{ab}	3.93^{abc}	4.54^{a}
G33-C33-O33	54.0^{d}	23.3^{abc}	15.7^{bc}	2.89^{a}	4.18^{a}	2.48^{de}
SEM	0.616	0.513	0.287	0.184	0.165	0.106
P-Value	<0.001	<0.001	<0.001	0.122	0.002	<0.001

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; ***. P<0.001; **. P<0.01; *. P<0.05; T. P<0.1; NS. P>0.1

- ***Long term effect on CH_4production and fermentation characteristics***

After five days of consecutive incubations, treatment effects were highly significant across treatments ($P < 0.001$) for total GP, CH_4, NH_3-N, total VFAs, and the CH_4:VFA ratio (Table 23). When garlic was added alone (G100), GP exceeded the control by 15%, CH_4 collapsed by 70%, NH_3-N fell by 16%, and VFA levels remained comparable to the control. Moreover, G10) uniquely sustained fermentation (GP and VFA levels close to control) while achieved 70 % CH_4 suppression and moderating ammonia reduction. Cinnamon (C100) and oregano(O100) both inhibited CH_4 formation by 90% and reduced NH_3-N by 30% and 40%, respectively; however, this came at the expense of fermentation, with total GP falling by 66% and 42% and VFAs by 81% and 74%, respectively. limiting their practical utility where microbial activity must be maintained (Table 24)

Binary blends of garlic–cinnamon (G50–C50) and garlic–oregano (G50–O50) achieved GP comparable to the control while suppressing CH_4 by 76 % and 47 %, respectively. The cinnamon–oregano (C50–O50) reduced CH_4 even further (-83 %) but at the expense of GP (-29 %). Notably, the ternary blend (G75–C12–O12) combined high GP (+15%) with maximal CH_4 abatement (-86 %), identifying it as the optimal ratio for sustaining GPwhile minimizing methanogenesis, and suggesting that the efficacy of additive combinations is time-dependent. The prolonged effect affected more the fatty acids shift (Table 24), likewise treatments produced distinctly different VFA fingerprints (all effects $P < 0.001$). The control fermentation was acetate-dominated (57.4 %), with substantial propionate (28.4 %) and moderate butyrate (11.3 %). Garlic alone (G100) continued to reduce acetate (–18%) and increase butyrate (+78%), while also causing excessive valerate accumulation and decreased propionate. Cinnamon (C100) enhanced acetogenesis, raising acetate by 32% at the expense of propionate (-59%), whereas oregano (O100) drove extreme butyrate increaseby 200% with nearly complete propionate suppression (–87%), resulting in a very high A:P ratio. Binary and ternary blends produced intermediate VFA profiles. The 50:50 garlic–cinnamon and garlic–oregano mixes reduced propionate by 55% and 33%, respectively, while doubling butyrate. The cinnamon–oregano blend retained high acetate (+20%, similar to C100) and high butyrate (+100%, similar to O100). Garlic-rich ternary mixtures (G75–C12–O12) maintained the VFA profile of pure garlic (G100). These results suggest that garlic's ability to redirect rumen fermentation toward propionate diminishes over prolonged incubation, instead sustaining acetate reduction and enhancing valerate production. Cinnamon continues to promote acetate formation and propionate suppression, whereas oregano favors butyrate synthesis.

Table 23: Overall fermentation parameters after five days of *in vitro* incubation of Garlic, Cinnamon, Oregano and their s combinations

	Total GP (ml)	CH_4 (%)	Total CH_4 (ml)	pH	NH_3-N (mg/dl)	VFAs (mmol/L)	CH_4:VFAs
CTL	97.2^{bc}	3.78^{a}	3.90^{a}	6.59^{d}	24.8^{a}	58.2^{a}	89.3^{a}
G100	111^{a}	1.13^{cde}	1.19^{c}	6.52^{e}	20.9^{ab}	60.0^{a}	89.7^{cd}
C100	32.8^{f}	1.09^{cde}	0.3^{e}	6.68^{ab}	17.5^{cde}	10.9^{e}	68.8^{ab}
O100	55.8^{e}	0.74^{de}	0.42^{e}	6.71^{a}	14.5^{de}	15.0^{de}	76.4^{bcd}
G50-C50	93.2^{c}	0.88^{cde}	0.92^{cde}	6.62^{cd}	17.8^{bc}	34.6^{c}	80.0^{bcd}
G50-O50	94.0^{bc}	2.20^{b}	2.08^{b}	6.62^{cd}	16.4^{cde}	45.4^{bc}	81.6^{ab}
C50-O50	69.2^{d}	0.89^{cde}	0.65^{cde}	6.66^{bc}	17.6^{cd}	22.7^{d}	83.8^{bcd}
G75-C12-O12	103^{ab}	0.55^{e}	0.54^{de}	6.54^{e}	18.3^{bc}	49.3^{ab}	85.4^{d}
G12-C75-O12	49.0^{e}	1.20^{cd}	0.59^{de}	6.59^{d}	17.3^{cde}	17.7^{de}	74.1^{bc}
G12-C12-O75	53.8^{e}	1.46^{c}	0.80^{cd}	6.63^{bcd}	14.4^{e}	19.6^{de}	74.6^{bc}
G33-C33-O33	73.5^{d}	1.37^{cd}	1.03^{cd}	6.60^{d}	16.9^{cde}	22.4^{d}	77.4^{ab}
SEM	3.48	0.221	0.186	0.017	1.104	3.75	8.23
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.003

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; ***. P<0.001; **. P<0.01; *. P<0.05; T. P<0.1; NS. P>0.1

Table 24: Fatty acids profile after 5 days of*invitro*incubation of Garlic, Cinnamon, Oregano and theirs combinations

	Acetate	Propionate	Butirate	Valerate	BCFAs	A:P
CTL	57.4d	28.4a	11.3d	1.15c	1.77a	8.05bc
G100	47.0f	24.8ab	20.2c	6.77a	1.24ab	1.91c
C100	75.7b	11.7d	8.8d	2.71c	1.02abc	6.66bc
O100	58.0d	3.5e	37.4a	0.890c	0.204cd	22.89ab
G50-C50	54.1de	12.7cd	26.4bc	5.89ab	0.902bc	4.78c
G50-O50	50.7ef	19.0bc	26.1bc	3.43bc	0.809bcd	2.99c
C50-O50	69.0c	2.8e	27.2bc	0.995c	0.000d	24.78a
G75-C12-O12	48.3ef	19.4b	24.1bc	7.35a	0.786bcd	2.53c
G12-C75-O12	88.3a	4.56e	5.73d	1.45c	0.000d	30.32a
G12-C12-O75	67.6c	7.51de	21.3bc	3.10c	0.494bcd	17.48abc
G33-C33-O33	66.8b	3.10e	28.0b	1.31c	0.749bcd	27.00a
SEM	2.215	2.294	2.588	0.879	0.295	5.680
P-Value	<0.001	<0.001	<0.001	<0.001	0.005	0.002

BCFA: Branched chain fatty acids; SEM: Standard error of the mean; ***. P<0.001; **. P<0.01; *. P<0.05; T. P<0.1; NS. P>0.1

- **Comparison between Day 1 and Day 5 effects of Garlic, Cinnamon, Oregano and their combinations**

All treatments exhibited the expected decline in fermentative activity between Day 1 and Day 5 (Figure16), but the slump magnitude varied markedly with additive type. In the control, total GP fell by 12 %, CH_4 by 31 %, NH_3-N by 30 %, and total VFAs by 35 % while pH remainedessentially constant. These shifts reflect the progressive decrease in the microbial activiyt not all microbial groups can proliferate *in vitro* during prologued periods. Garlic (G100) was unique in maintaining and even slightly increasing GP (+2 %) over five days, while preserving its anti-methanogenic effect, with reduced deamination effect by 29 %, and reduced VFAs production by 33 %. Cinnamon (C100) and oregano (O100), by contrast, both achieved the strongest CH_4 inhibition by day 5 even more than results obtained after 1 day incubation (from-46%to -71%) and (from -6% to -80%) respectively but at the expense of drastic reductions in gas and VFAs (-84%), as well as pronounced NH_3-N declines (from-15% to -30%) and (from -10% to -40%). This illustrates that extreme anti-methanogenic potency can severely impair overall fermentative performance and suggests that, like most essential oils, these additives may require extended incubation to fully manifest their effects. Some blends provide a means of balancing these effects. For example, the garlic–cinnamon binary (G50–C50) maintained CH_4 suppression above 70 %, while the garlic–oregano pair (G50–O50) exhibited a lower antimethanogenic effect (from -74 % to -46 %). Both mixtures preserved total GP within 5 % of the control and remarkable VFA losses (-57 % and -44 %, respectively). Notably, the cinnamon–oregano blend (C50–O50) showed increasingly pronounced effects by Day 5 across all parameters, likely reflecting a delayed yet potent activation of oregano's bioactive compounds. In contrast, the ternary mixture (G75–C12–O12) proved optimal as it sustained GP at control levels, delivered superior antimethanogenic and deaminative results, and limited VFA decline to just 15 %. Overall, these data confirm that garlic-dominant combinations can preserve fermentative performance while achieving substantial CH_4 inhibition, whereas treatments concentrated on cinnamon or oregano incur extreme anti-methanogenic effects at the cost of overall microbial activity and fermentation products.

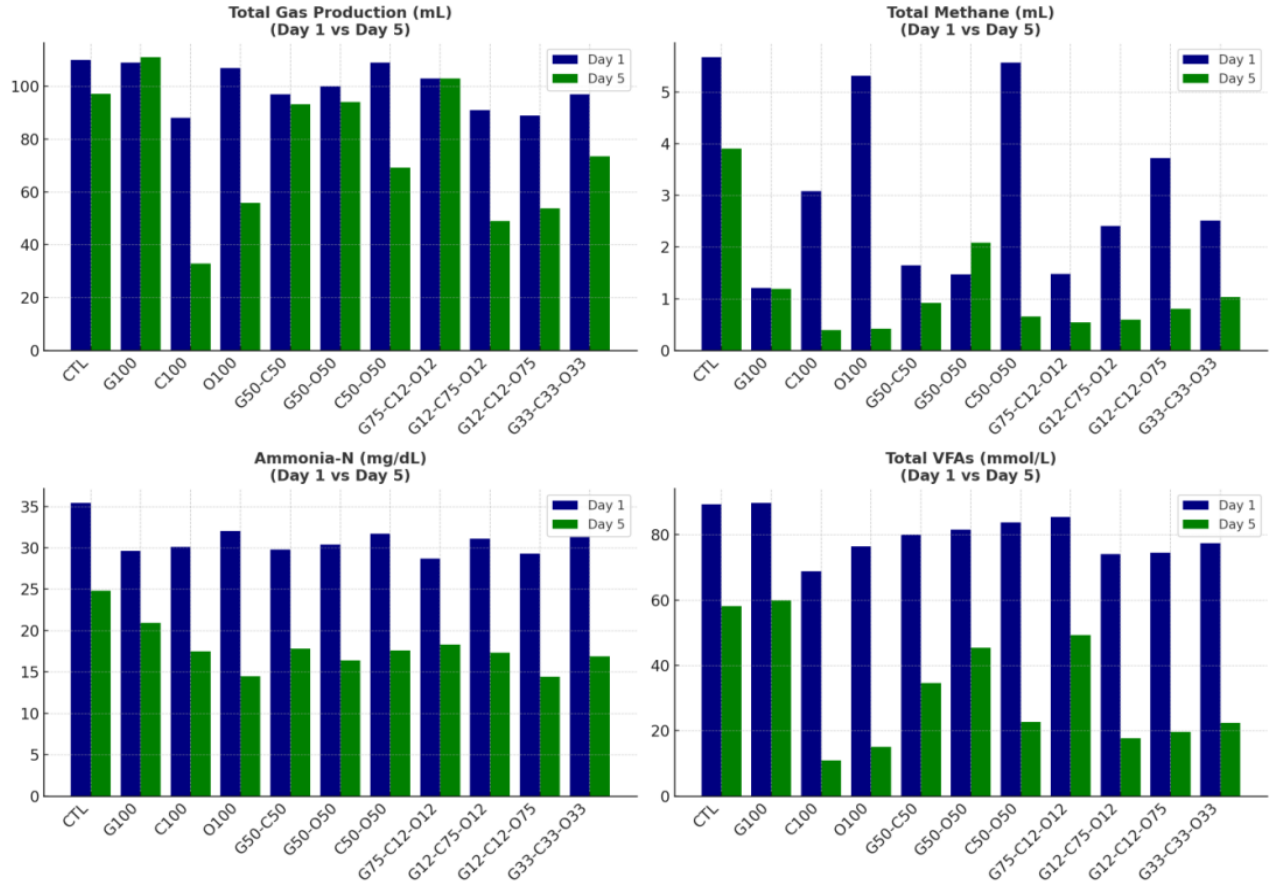


Figure 16: Fermentation parameters comparison between Day1 and Day5 *in vitro* incubation with Garlic, Cinnamon, Oregano, and their combinations.

Regarding the VFA profile between Day 1 and Day 5 (Figure 17), we can note that Garlic (G100): continues to decrease acetate by Day 5. Cinnamon (C100): conversely, stimulates acetate production, which increases significantly between Day 1 and Day 5. Oregano (O100): barely alters the acetate proportion over time maintaining stable profile. For Propionate and Butyrate, Garlic: retains a modest propiogenic capacity because a slight propionate decrease is noted by Day 5, while butyrate rises moderately. Cinnamon and Oregano: both cause a strong reduction in propionate, in favor of a marked increase in butyrate. Garlic-dominant combinations (G75–C12–O12, G50–C50, and G50–O50): generally, follow the pure garlic pattern, though effects are attenuated by Day 5. Nevertheless, these blends maintain the highest propionate levels. Notably, (G50–O50) appears to mitigate oregano's strong anti-propionate effect. Concerning valerate, an excessive accumulation is observed with pure garlic and mixtures containing 50 % or 75 % garlic, suggesting a garlic-specific secondary metabolic pathway. Lastly, BCFAs proportions decrease under all tested conditions. This reduction is especially pronounced for pure cinnamon and its blends (C100, C50–O50, and G12–C75–O12). In summary, after 5 days of *in vitro* incubation, garlic is characterized by a strong acetate reduction and valerate accumulation while maintaining relatively high propionate. Cinnamon, on the other hand, promotes acetate, whereas oregano shifts metabolism heavily toward butyrate without changing acetate. Garlic-dominant blends partially balance these effects, yielding intermediate profiles but still high in butyrate and valerate.

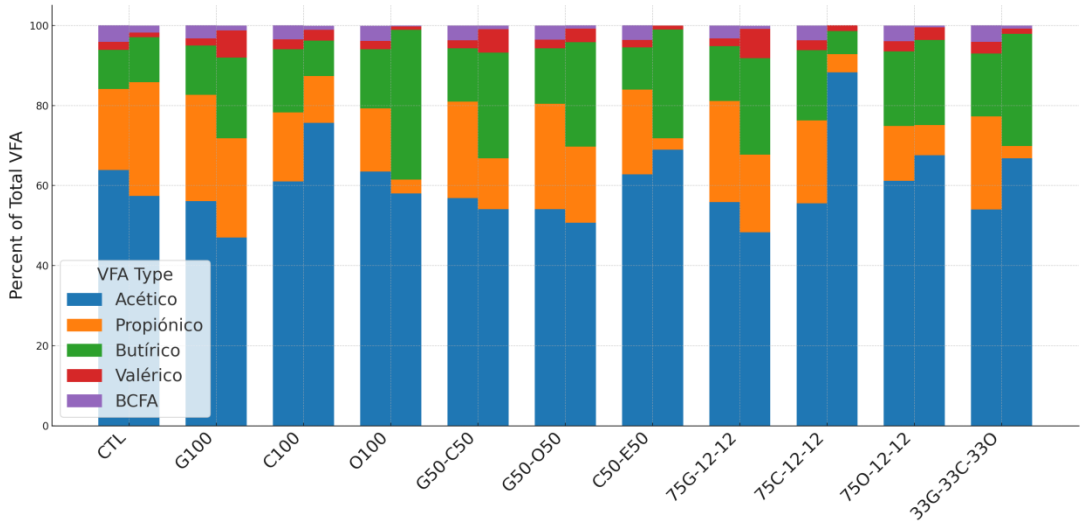


Figure 17: Fatty acids profil comparison between Day1 and Day5 *in vitro* incubation with Garlic, Cinnamon, Oregano, and their combinations.

6 DISCUSSION

6.1 Effect of Garlic

Garlic extracts (*Allium sativum*) representone of the most sulfur-rich plant products, supplying 5–37 g S/kg DM (Kamra et al., 2012). The primary bioactive molecule, allicin, is highly unstable and quickly converted into secondary sulfur compounds like diallyl disulfide (DADS). Numerous studies show that garlic extracts and oils can exert modulatory effects on the rumen fermentation: they enhance nutrient digestibility, lower protozoal counts, and depress enteric CH_4 emissions, along with notable shifts in the rumen microbiota(Hart et al., 2008; Wanapat et al., 2008; Yang et al., 2010).

In batch cultures, the antimethanogenic response is highly dose- and diet-dependent. When garlic powder, whole-bulb filtrate, purified DADS, or garlic oil were added to substrates ranging from corn silage (Vargas et al., 2023), 100% forage (Kim et al., 2020) 70:30 (Busquet, Calsamiglia, Ferret, Cardozo, et al., 2005), 60:40 (Kongmun et al., 2010), or 50:50 (Patra & Yu, 2015)forage:concentrate ratios, CH_4 yields fell by 10–90%. The inocula were sourced from buffalo (Patra et al., 2006), cattle(Roque et al., 2019), or sheep (Barreto-Cruz et al., 2023), confirming that both dose and basal diet modulate the influence of garlic constituents on CH_4 production and VFA profiles. Continuous-culture experiments reinforce these findings: garlic additives reduced energy losses as CH_4 and N-losses as NH_3-N, thereby improving overall nutrient-use efficiency (Cardozo et al., 2005; Ding et al., 2023; Sari et al., 2022). In our experiments (1 and 3), the garlic extracts Garlic-1 and Garlic-2 contained 5.65% and 4.87% diallyl disulfide (DADS), respectively. Adding either extract did not affect total GP or the total concentration of VFA, yet it consistently shifted the VFA pattern: acetate and BCFA decreased, whereas propionate and butyrate increased. This shift is consistent with previous work linking garlic-derived organosulfur compounds to better fiber degradation and more efficient use of reducing equivalents, possibly via stimulation of anaerobic rumen fungi (Klevenhusen et al., 2011; Saro et al., 2018). Additionally, in the rumen, Gram-positive bacteria are typically acetate- and butyrate-producers, whereas Gram-negative bacteria are often associated with propionate production (Stewart et al., 1997) suggesting that garlic extracts enhance gram-negative bacteria. Comparable effects have been reported for garlic oil, purified DADS, and allyl mercaptan, all of which lower the molar proportion of acetate while increasing propionate and butyrate in batch cultures, thereby improving the energetic efficiency of rumen fermentation (Ahmed et al., 2021; Busquetet al., 2005). Both extracts reduced CH_4 and NH_3–N in a clear dose-dependent manner, with Garlic-1 invariably giving the stronger response. The pattern fits the well-known stoichiometric link between fermentation end-products and hydrogen disposal: higher acetate is positively, and higher propionate negatively, correlated with CH_4 formation because propionate synthesis is a major hydrogen sink that competes with methanogenesis (Ungerfeld, 2015, 2020; Vargas et al., 2020). Moreover, the suppression of CH_4 is most plausibly direct, via the toxicity of garlic organosulfur compounds, particularly diallyl sulfide and allicin, toward methanogenic archaea. These molecules react with sulfhydryl groups, inactivating key enzymes and reducing the abundance of the dominant family *Methanobacteriaceae*(Eger et al., 2018; Martin et al., 2010)The concurrent fall in NH_3–N, despite unchanged total VFA, points to more efficient NH_3–N capture for microbial protein synthesis (Bach et al., 2005) and/or a direct antagonism of proteolytic bacteria. Consequently, Garlic-1 was carried forward to a five-day consecutive fermentation to test the persistence of its effects.

In both runs (Experiment 2 and Experiment 4), the same response pattern re-emerged: GP was higher than the control, CH_4 and NH_3–N were consistently lower, total VFA concentration remained unchanged, and the VFA profile again shifted from acetate toward propionate and butyrate. Valerate

also increased, indicating that surplus reducing equivalents were being oriented into valerate synthesis. While previous studies highlight a rise in propionate and butyrate, typically paired with a fall in acetate, the shift of other VFAs, including valerate, was less predictable (Busquet, Calsamiglia, Ferret, Carro, et al., 2005; Chaves et al., 2008). Notably, marked valerate increases after garlic or organosulfur supplementation have been documented by Ahmed et al. (2021) and Busquet et al (2005)

Throughout our experiments, we observed that at a constant inclusion rate of 12 mg/g DM, Garlic-1 reduced CH_4 production by 83% in Experiment 1, 78% in Experiments 2 and 4, and 56% in Experiment 3 after 24 h of incubation; and 69% in the five-day consecutive run. All experimental conditions were identical except for the source of rumen fluid, so this spread most likely reflects inter-animal differences in methanogen abundance and overall microbial community composition(Mateos et al., 2013). Additionally, the degree of suppression we observed exceeds the values usually reported for pure diallyl disulfide, implying that Garlic-1 may contain other garlic-derived bioactive compounds that act synergistically with DADS. Because dietary garlic constituents are rapidly metabolized to a suite of secondary sulfur compounds, such synergism probably accounts for the exceptional antimethanogenic effect recorded. These findings show that garlic extracts, particularly Garlic-1, due to its multifaceted effects on CH_4, NH_3–N, and fermentation profile consistency across time are promising rumen additives.

Garlic products have generally shown positive effects on feed intake, nutrient digestibility, and growth performance in ruminants when included at low levels (4–60 mg/kg DM) over periods ranging from 21 days to 6 months (El-Naggar & Ibrahim, 2018; Kongmun et al., 2010; Zhong et al., 2019; Zhu et al., 2021). Nevertheless, other studies have reported no significant effects under similar conditions (Bampidis et al., 2005; Ikyume et al., 2020). Only a limited number of *in vivo* studies have examined the impact of garlic or its sulfur-containing constituents on milk yield and quality in dairy cows (Blanch et al., 2016; Oh et al., 2017; van Zijderveld et al., 2011).Furthermore, data on the effects of garlic supplementation on the sensorial and rheological properties of milk in ruminants remain scarce. In humans, volatile metabolites derived from garlic have been detected in milk just a few hours after garlic-clove ingestion, consistently modifying its sensory profile (Scheffler et al., 2019). Similarly, we hypothesized that the inclusion of garlic, sulfur-rich compounds in ruminant diets could alter the sensory characteristics of milk, potentially affecting consumer acceptance of the final product. To address this concern, we evaluated combinations of garlic with other additives at different inclusion rates in order to determine which formulation and dose could preserve the functional efficacy of garlic while minimizing its potential negative sensory effects, particularly odor transfer to animal-derived products.

6.2 Effect of Essential Oils

Essential oils (EOs) derived from various plant species vary in their chemical structures and bioactive components (Burt, 2004). When added to ruminant diets, EOs have demonstrated the ability to modify digestion, fermentation patterns, microbial populations, and methanogenesis within the rumen (Calsamiglia et al., 2007; Cieslak et al., 2013). Their selective inhibition of specific microbial groups, including methanogens, protozoa, and hyper-ammonia-producing bacteria, is regarded as a primary mechanism for modulating rumen fermentation (Calsamiglia et al., 2007). While EOs are active against both Gram-positive and Gram-negative bacteria, the outer membrane of Gram-negative bacteria offers some resistance, making them generally less susceptible(Davidson & Naidu, 2000). As a result, shifts in bacterial composition may suppress certain fermentative pathways while favouring Gram-negative species (Klevenhusen et al., 2012).

- ***Eugenol***

Eugenol is a phenylpropanoid compound that constitutes up to 85% of clove (*Syzygiumaromaticum*) oil (Hu et al., 2018). It exhibits broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria (Dorman & Deans, 2000; Walsh et al., 2003). In our first incubation (pH = 6.73), eugenol did not produce any measurable effect on GP, CH_4, NH_3–N, or VFA profiles. In contrast, under slightly more acidic conditions in Experiment 2 (pH = 6.59), the same dose of eugenol significantly reduced CH_4 and NH_3–N concentrations and altered VFA proportions. These contrasting outcomes are in agreement with previous studies that highlight the pH- and dose-dependence of eugenol's effects(Busquet et al., 2005; Cardozo et al., 2005).

Cardozo et al. (2005) demonstrated that eugenol at concentrations of 0.3, 3, and 30 mg/L increased total VFA production and reduced the molar proportion of propionate at a low pH (5.5), whereas no significant effects were observed at higher pH (7.0). (Yang et al., 2010) similarly reported that eugenol, or EO blends rich in eugenol, markedly inhibited proteolysis and amino acid deamination *in vitro* under low pH conditions. This suggests that eugenol may be more effective under the acidic rumen environments typical of high-concentrate diets in beef cattle systems. Nevertheless, in vivo studies have shown mixed results. For example, eugenol supplementation in beef cattle (Yang et al., 2010) and dairy cows (Benchaar et al., 2012) did not significantly alter NH_3–N levels, contrary to observations by (Cardozo et al., 2006).

In our consecutiveincubation (Experiment 2), the GP on day 1 remained unchanged. However, CH_4 and NH_3–N were moderately reduced (-15% and -6%, respectively), while a distinct shift in fermentation was already evident: total VFAs decreased (-10%), propionate dropped (-13%), and butyrate increased (+16%). Similar trends were observed by Castillejos et al.(2006) who reported reduced propionate and increased butyrate with 500 mg/L of eugenol. Busquet et al. (2005) also noted increased butyrate and reduced NH_3–N and BCFAs, consistent with reduced amino acid deamination (Allison & Bryant, 1963). In our study, the decrease in NH_3–N on day 1 remained limited (-6%) and the BCFA reduction (-9%) was not statistically significant.

Eugenol produced the strongest suppression of GP among all EOs tested: GP was reduced by 31% on Day 2, with only partial recovery by Day 5 (-28% relative to control). This pattern coincided with major reductions in CH_4 (-62%), NH_3–N (-23%), and total VFAs (-24%), along with increased pH. The most notable shift was observed in propionate (-49%) and butyrate (+120%) by Day 5, indicating substantial changes in fermentation pathways. Accordingly, an increase in the proportion of butyrate appears to be an indication that the concentration of EO is higher than optimal to improve rumen energy efficiency (Joch et al., 2016, 2019).

Several mechanisms may explain the observed effects. First, the need for an adaptation period suggests that eugenol's activity emerges only after selective shifts in the microbial community. Second, the decreaseof propionate molar proportion supports the hypothesis that eugenol preferentially inhibits Gram-negative, propionate-producing bacteria (Chericoni et al., 2005). Third, the observed reductions in CH_4 and NH_3–N may reflect a combination of reduced bacterial activity due to progressive and recurrent dosage of eugenol during consecutive incubation. This is in line with findings from Chaves et al. (2008), who reported that declines in CH_4 and VFAs may indicate impaired feed digestion. The dailyrenew of the incubation media over time may have limited microbial recovery and reinforced the antimicrobial action of the additive. This is particularly relevant given that rumen microbes have been

shown to adapt or degrade essential oils with prolonged exposure (Benchaar et al., 2008; Benchaar and Greathead, 2011).

- ***Cinnamon***

Cinnamon bark and leaves represent a natural source of essential oils for various applications (Kallel et al., 2019). Among the 250 species within the genus *Cinnamomum*, *C. verum* and *C. cassia* are most commonly used in essential oil production. The main bioactive compounds found in cinnamon oil are cinnamaldehyde, eugenol, and camphor and they are known for their potent antimicrobial and antioxidant properties (Lewa & Gugule, 2022; Schmidt et al., 2006; R. Wang et al., 2009) The composition of these oils depends on the species, plant part, and extraction method considered (Abd El-Hack et al., 2020).

Cinnamaldehyde, the major component of cinnamon oil, disrupts microbial cytoplasmic membranes and inhibits key enzymes, affecting microbial growth and metabolism (Burt, 2004; Gill and Holley, 2004; Ouwehand et al., 2010). It also interferes with rumen proteolytic bacteria either directly or by reducing substrate accessibility. In our study (Experiment 3), high doses of cinnamon oil (100–200 mg/g DM) significantly inhibited fermentation, as evidenced by reduced GP, total VFAs, and increased pH. These results suggest excessive antimicrobial activitywhen used above 100 mg/d DM. This suppression was accompanied by reduced CH_4 emissions (up to -98%, $P < 0.001$) and NH_3–N concentrations (up to -33%). Lower doses (25 and 50 mg/g DM) did not significantly affect GP, CH_4, NH_3–N, pH, or VFA profiles. These findings suggest a narrow optimal dose range for cinnamon oil. These results are consistent with prior studies showing that high doses of cinnamon oil can inhibit fermentation. Khorrami et al. (2015) observed similar reductions in GP and VFAs at 1000 mg/L after 24 hours, and Macheboeuf et al. (2008) reported CH_4 reductions of 76% at 400 mg/kg. Cinnamon's antimethanogenic effect is likely due to its direct inhibition of methanogens or associated enzymes (Calsamiglia et al., 2007; Chaves et al., 2008; Cieslak et al., 2013). Macheboeuf et al. (2008) also found that whole cinnamon oil was more effective than pure cinnamaldehyde, possibly due to synergistic effects with other components such as eugenol. Similarly, Busquet et al. (2006) demonstrated that cinnamon oil reduced NH_3–N by approximately 50% at 3000 mg/L, while lower doses had minimal effects. In our study, increasing cinnamon concentrations progressively reduced NH_3–N, likely reflecting decreased proteolytic and peptidolytic activity.

In vivo, CH_4 mitigation by cinnamaldehyde showed less consistent results, but reduced microbial protein synthesis has been reported(Chapman et al., 2019). This supports the idea that EOs may affect microbial efficiency more than methanogenesis directly(Benchaar, 2016; Benchaar et al., 2008).EOs tend to be more effective against Gram-positive bacteria, many of which produce acetate (A. K. Patra & Yu, 2012), whereas gram-negative, propionate-producing species are generally more resistant (Holley & Patel, 2005). The effects of cinnamon oil on VFA profiles have been inconsistent in the literature. For example, Busquet et al. (2006) reported acetate increases and propionate decreases, whereasKhorrami et al. (2015) and Vakili et al. (2013)observed the opposite. These variations may result from differences in diet, EO composition, or cinnamon chemotypes. The acetogenic shift in our study, similar to that observed with eugenol, suggests that our cinnamon oil likely contained both cinnamaldehyde and eugenol.

In Experiment 4, an intermediate dose (80 mg/g DM) was selected for the consecutive batch culture trial to assess long-term effects of cinnamon oil. A marked GP inhibition occurred by Day 2 (-66%), with limited recovery by Day 5 (-60%) in comparison to CTL. Nevertheless, between Days 3 and 4, GP

declined slightly (-5%), followed by partial recovery (+53%) by Day 5, suggesting microbial adaptation to the *in vitro*conditions. Busquet et al. (2005) and Cardozo et al. (2004) previously reported that microbial adaptation to EOs can occur within one week. Similar trends have been observed *in vivo*(Geraci et al., 2012; Vakili et al., 2013; Yang et al., 2010)..At Day 1, cinnamon oil (C100) reduced CH_4 by 45% ($P < 0.001$) and NH_3–N by 15%, with 20% reductions in GP and VFAs ($P < 0.05$) consistent with (Calsamiglia et al., 2007; Macheboeuf et al., 2008). By Day 5, CH_4 inhibition reached 90% and NH_3–N reduction stabilized at 30%. However, this was accompanied by substantial losses in fermentation: GP dropped by 66% and VFAs by 81%. As described for eugenol, these effects may result from continuous dilution of inoculums added to high doses used in comparison to previous studies. Future studies could evaluate the long-term effects of lower doses (25–50 mg/g DM) in semi-continuous Rusitecsystems to assess whether they give similar results.

- ***Oregano***

Origanum vulgare is an aromatic perennial plant of the Lamiaceae family, widely used in animal nutrition as a natural source of phytochemicals. Its major bioactive components; carvacrol and thymol, are primarily responsible for its broad-spectrum antimicrobial properties (Kolling et al., 2018). Supplementation with oregano has been associated with improved fermentation, reduced CH_4emissions (Hristov et al., 2013), enhanced fiber digestibility (Jiao et al., 2021), and shifts in rumen microbial populations (Zhou et al., 2019, 2020). The strong antimicrobial effects of thymol and carvacrol are linked to their phenolic hydroxyl groups (Calsamiglia et al., 2007; Benchaar and Greathead, 2011). In our study (Experiment 3), increasing doses of oregano oil induced a dose-dependent inhibition of ruminal fermentation. A linear decrease in GP, CH_4, total VFAs, and NH_3–N concentration was observed, along with a rise in pH. At 50 mg/g DM, GP dropped by 32%, CH_4 by 45%, and VFAs by 14% without altering the VFA profile, suggesting this may be close to the optimal dose for balancing antimethanogenic effects while maintaining fermentation stability. At higher doses (up to 200 mg/g DM), fermentation was strongly inhibited, with reductions of up to 83% in GP and 63% in VFAs, resembling the patterns observed for cinnamon oil.These findings align withSoltan et al. (2011) , who showed that high carvacrol levels (20 mL/L) significantly reduced CH_4 and GP while decreasing organic matter degradability. On the contrary, moderate doses (5–10 mL/L), however, appeared promising for mitigating CH_4 without negatively impacting digestibility. In our study, pH increased with rising oregano doses, likely due to reduced GP and VFA production, consistent with (Lin et al., 2012) and Patra and Yu (2012).It is important to note that effective EO concentrations *in vitro* often exceed realistic *in vivo* inclusion levels, where high doses may reduce palatability or pose toxicity risks (Benchaar and Greathead, 2011). The EO composition of oregano varies with environmental and botanical factors (Benchaar et al., 2009), complicating comparisons among studies. For instance,(Olijhoek et al., 2019) and (Benchaar, 2020) reported no CH_4 reduction in cows supplemented with 500mg/kg DM of oregano EO or carvacrol. (Stefenoni et al., 2021) also observed no CH_4 reduction using 1.7% oregano leaf in TMR. In contrast,Kolling et al. (2018) achieved CH_4 reduction with 560mg oregano extract/kg DM, and Hristov et al. (2013) recorded in dairy cow up to 36% CH_4 inhibition with 500 g/day of oregano leaves containing 1.5% EO. These discrepancies highlight that oregano's efficacy depends on EO composition and experimental context.

To assess long-term effects, a consecutive batch culture (Experiment 4) using the 50 mg/g DM dose was conducted as it was considered as the optimum dose based on Experiment 3. Supplementation with oregano alone (O100) promoted an initial drop in GP by 59% in Day 1, followed by modest recovery from Days 3 to 5, suggesting microbial adaptation (Busquet et al., 2005; Cardozo et al., 2004). CH_4 and GP remained comparable to CTL, while VFAs declined by 14%. Fermentation shifted: propionate fell

by 22%, butyrate rose by 52%, and the acetate-to-propionate (A:P) ratio increased in consistency with (Castillejos et al., 2008). By Day 5, CH_4 was reduced by 90% and NH_3–N by 40%, but fermentation was markedly impaired: GP dropped by 42%, VFAs by 74%, and propionate by 87%, while butyrate increased by 200%, sharply raising the A: P ratio. These findings contrast with Castillejos et al. (2008), who reported more stable VFA profiles under continuous culture. The dose used in our study may have exceeded the adaptive capacity of rumen microbeswhen daily supplemented during 5 consecutive days. The pronounced suppression of propionate supports the hypothesis that oregano EO selectively inhibits Gram-negative, propionate-producing bacteria similar to the mechanisms proposed for eugenol and cinnamon oil.

6.3 Effect of Polyphenols

Tannins are polyphenolic compounds with a wide range of molecular weights, known for their ability to bind natural polymers such as proteins and carbohydrates (Mueller-Harvey, 2006). Based on their chemical structure, tannins are classified into two main groups: hydrolysable tannins (HT), which are polyesters of gallic acid and various sugars, and condensed tannins (CT), which are polymers of flavonoid units (McSweeney et al., 2001). Although the mechanisms by which tannins mitigate enteric CH_4 emissions are not fully elucidated, they appear to involve multiple pathways, including a reduction in fiber digestibility thus limiting H_2availability for methanogenesisand a direct inhibitory effect on methanogenic archaea (Tavendale et al., 2005). HT can also bind to microbial cells and dietary proteins, thereby modulating microbial activity and reducing ruminal proteolysis, which alters N metabolism (Tan et al., 2021). Some studies report that HT are more effective than CT in reducing CH_4 emissions while preserving nutrient digestibility (Jayanegara et al., 2015; Yanza et al., 2021). However, most studies report the opposite, highlighting the greater consistency and long-term efficacy of CT, whereas HT tend to be less stable and more rapidly degraded in the rumen (Patra & Saxena, 2011).In our study, most of the polyphenols tested were hydrolysable tannins rich in gallic acid (GA), derived from various plant materials, except Polyphenols 13 and 14, which are flavonoids from *Scutellariabaicalensis*. Both HT and CT have been reported to reduce N degradation and improve N utilization efficiency *in vitro* (Chen et al., 2021) and *in vivo*(Norris et al., 2020). In our experiments, the response to GA-containing extracts varied: while some treatments had no significant effect, others resulted in decreased pH and NH_3–N levelsfindings consistent with Aboagye et al. (2019),Getachew et al. (2008), and (Manoni et al., 2024).

A recurring effect across treatments was an increase in GP. However, the literature on GA's impact on GP is mixed. Getachew et al. (2008) reported that GA supplementation (up to 150 mg/g DM) increased GP after 72 h, whereas (Geerkens et al., 2013) observed suppressed fermentation after 24 h with similar doses. In our case, the increased GP is likely due to the relatively low inclusion levels (up to 12 mg/g DM), which may have improved substrate fermentationwithout exerting strong antimicrobial effects. This is supported by Wei et al. (2019), who found that GA at 20 mg/g DM slightly increased GP without affecting CH_4 or NH_3–N, suggesting that GA can increase feed degradation in the rumen. The amount of GA in the polyphenol varied across extracts (Polyphenols 1 (9.96%), Polyphenol 2 (0.9%), Polyphenol 6 (5.9%), and Polyphenol 7 (2.6%)) therefore the amount of each polyphenol source was normalized in order to provide the same amount of GA across treatments.Aboagye et al. (2019) showed that 1.5% pure GA reduced CH_4 and NH_3–N, emphasizing the role of compound purity and matrix effects in raw material.This may explain why Polyphenol 10 (49% GA), which was supplied at low amounts given its high GA content, failed to increase GP in comparison to the polyphenol sources.

Polyphenol 6 (5.6% GA) showed no significant effects at 4 mg/g DM (Experiment 1), but at 12 mg/g DM (Experiment 3), it increased GP (+23%), reduced pH, and shifted the VFA profile toward higher acetate and lower propionate and butyrate, similar to observations by Manoni et al. (2024). While NH_3–N remained stable, BCFA levels declined, indicating possible modulation of amino acid metabolism. This suggests Polyphenol 6 may enhance fibrolytic activity and raise the acetate-to-propionate (A: P) ratio. At moderate doses (~3 mg/g DM); it reduced CH_4 by 21% without compromising VFA production.

Polyphenols 13 and 14 were derived from *Scutellariabaicalensis*, a medicinal plant from the Lamiaceae family. The roots of this plant are rich in flavonoids such as baicalin, baicalein, and scutellarincompounds known for their antimicrobial, antioxidant, and anti-inflammatory activities. Baicalin is the most abundant flavonoid and is stable in acidic media but degrades under alkaline conditions(Zhou et al., 2019). Given the broad-spectrum antimicrobial activity of *S. baicalensis*, we hypothesize that the low doses of Polyphenols 13 and 14 used in our study did not inhibit but rather modulated the rumen microbiota, favoring fermentative bacteria. This may explain the slight increase in GP, reduction in pH, and a shift toward higher acetate and lower propionate levels. These effects are consistent with a response, where low doses enhance microbial activity (Calabrese, 2013).

6.4 Effect of Green Seaweed

To date, more than 21 seaweed species have demonstrated the potential to reduce CH_4 emissions *in vitro*(Abbott et al., 2020). Among these, the red seaweed *Asparagopsistaxiformis* has shown the highest efficacy, achieving nearly complete CH_4 inhibition at inclusion levels up to 16.7% of organic matter (OM). *In vivo* studies have also reported that including 5% *A. taxiformis* in dairy cow diets reduced CH_4 emissions by up to 95% without adverse effects on fermentation. This potent antimethanogenic activity is attributed to halogenated CH_4 analogs like bromoform, which inhibit key enzymes in methanogenesis (Machado et al., 2016). While red seaweeds have been widely studied, some green seaweeds, such as *Cladophora patentiramea* and *Ulva* spp., have also demonstrated moderate CH_4 mitigation (>50%) *in vitro*, though often at the cost of reduced GP (Machado et al., 2014). Green algae, especially *Ulva* and *Enteromorpha* species, have fewer secondary metabolites (<300 identified compounds) than red or brown algae (Abbot et al., 2020). However, they are rich in unique polysaccharides that can serve as carbon sources for microbial biosynthesis of organic acids, alcohols, and other fermentation products. These structural carbohydrates may partially explain the modest increases in GP or altered fermentation profiles sometimes observed.

In our study two sources of green seaweed were evaluated *in vitro*. Given the high inclusion rate tested (up to 20% in DM), these seaweeds were used as a partial replacement of the festuca hay. In Experiment 1, supplementation with Green Seaweed 1 and 2 did not affect pH, CH_4 emissions, or total VFAs, suggesting a similar supply of fermentable nutrient than observed for festuca hay in the CTL treatment. However, both seaweeds led to a dose-dependent increase in NH_3–N and a slight increase in valerate concentration. These changes suggest stimulation of deamination and proteolysis processes and subtle modulation of minor VFAs, without direct antimethanogenic action. Our results are consistent with Roskam et al. (2022), who reported that incubating *Ulva* spp. at 10 g/kg DM in a semi-continuous culture had no effect on CH_4 but increased NH_3–N. Although a trend to decrease GP was noted in our experiment, the effect was not statistically significant. The absence of CH_4 reduction suggests that the tested doses of green seaweed did not exert a strong enough inhibitory effect on methanogenesis. The increased NH_3–N concentration may be linked to a higher rate of protein deamination or the presence of nitrogenous compounds in the algal biomass. Taken together, these findings indicate that while green

seaweeds such as *Ulva* spp. can modulate N metabolism in the rumen, their use as antimethanogenic agents may require either: higher doses, different species, or synergy with other additives.

After analyzing the effects of individual additives on rumen fermentation and CH_4 production, it becomes relevant to evaluate the potential effects when these compounds are supplemented in combination. Additive interactionswhether synergistic, neutral or antagonisticcan significantly influence their overall efficacy. In particular, understanding whether combining certain bioactive compounds enhances or counteracts their individual effects can inform more effective mitigation strategies. The following section explores these interactions based on the results obtained from Experiments 2 and 4, focusing on GP dynamics across incubation time.

6.5 Effect of mixtures

Several studies have demonstrated that garlic products offer a range of biological benefits to ruminants (Ding et al., 2023; Horton et al., 1991; Zafarian and Manafi, 2013). However, there is a lack of comprehensive and systematic information on how garlic products influence ruminant production systems. Moreover, sensory perception plays a critical role in feed acceptance and intake in herbivores, and the strong odor of garlic may negatively affect palatability or be transferred to animal-derived products (Cannas et al., 2009). Based on these considerations, we proposed that combining garlic with other additives of different origins rates could maintain or even enhance its antimethanogenic and propiogenic effects while mitigating undesirable sensory characteristics. To test this, three additives were selected from the previous dose–response experiments (Experiments 1 and 3) and evaluated in combination with garlic in Experiments 2 and 4. The primary objective was to identify synergistic or additive interactions capable of preserving the functional properties of garlic while minimizing its negative impacts, particularly those associated with odor. In parallel, combinations among the other selected additives were also evaluated to investigate potential synergistic effects.

- **Synergistic effects between garlic extract, polyphenols and eugenol**

Plante Pure garlic extract alone (G100) consistently produced the highest GP across all incubation times, with GP at day5 values surpassing those recorded on day 1. This reinforces the idea that garlic, at the tested dose, effectively stimulates fermentation, likely due to its modulatory effect on the ruminal microbiota.

Although the binary and ternary mixtures performed slightly below the (G100), their values remained close (Figure 18). Binary blends, specifically (G50–E50 and G50–P50), retained the antimethanogenic and propiogenic properties of pure garlic. Notably, the G50–P50 combination yielded the largest reduction in total VFA concentration (-12%), exceeding the effects observed with garlic or polyphenols alone, despite maintaining relatively high GP. This suggests that polyphenols may partially attenuate garlic's stimulatory effects on fermentation. Additionally, a decrease in NH_3–N concentration was observed in these combinations compared to polyphenol-only (P100) or eugenol-only (E100) treatments, suggesting a potential synergistic deamination effect when garlic is combined with these additives. The binary combination (G50–E50) improved GP compared to eugenol alone, while G50–P50 performed slightly lower than polyphenols alone in terms of GP. Nevertheless, both blends were effective in reducing CH_4 and NH_3–N. In contrast, the (E50–P50) blend showed minimal efficacy, with no notable improvement in fermentation or gas-related parameters, highlighting a possible antagonistic interaction. Bassolé& Juliani, (2012) indicated that phenolic compounds tend to have additive effects, while synergistic or antagonistic effects would occur with other chemical compounds and vary

depending on the microbial ecosystem. Ternary blends exhibited dose-dependent effects, especially for combinations (G75–E12–P12) and (E75–G12–P12). The ternary blend (P75–G12–E12) showed improved VFA production and more pronounced reductions in CH_4 and NH_3–N, indicating a favorable synergy. These results align with Cobellis et al (2016) suggesting that minor EO compounds may play an important role in determining anti-methanogenic activities.

The equal-dose ternary combination (G33–E33–P33) resulted in a smoother and more stable fermentation pattern over time. This blend also achieved an intermediate reduction in CH_4 emissions (-63%) without disturbing VFA demonstrating that balanced inclusion rates can promote both fermentative stability and CH_4mitigation.As a result, this ternary combination should be further evaluated as one of the most promising findings of this study.

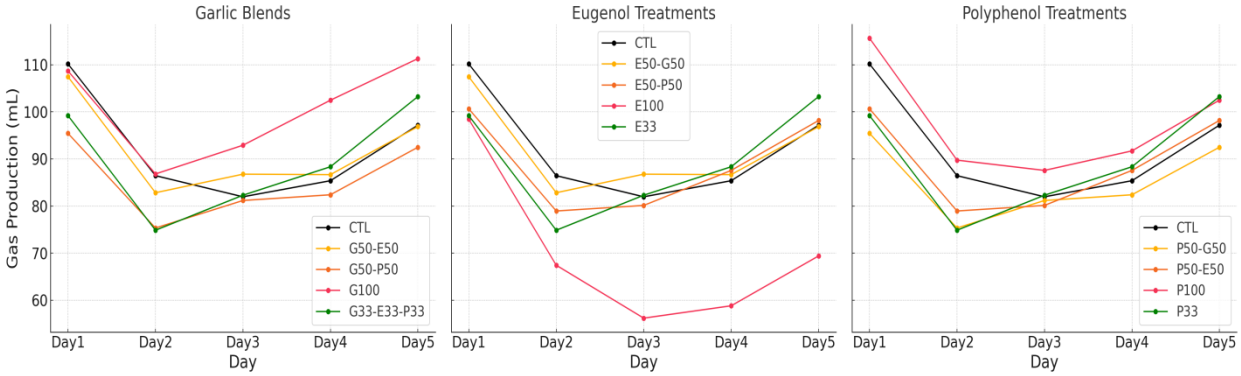


Figure 18: Effects of Single and Combined Additives on Gas Production: Exploring Synergistic and Antagonistic Interactions

- **Synergistic effects between garlic extract, oregano and cinnamon**

In this experiment, garlic was mixed with two essential oils cinnamon and oregano. Binary combinations such as (G50-C50) and (G50-O50) showed a temporary decline in GP on day 2, followed by a gradual recovery, indicating a transient inhibitory effect followed by microbial adaptation. These blends also led to a reduction in CH_4 emissions and improved VFA profiles, suggesting a potential synergy between the two additives and garlic. Notably, the same propiogenic shiftcharacteristic of garlic was maintained, resulting in a lower A: P ratio, which implies that garlic may mitigate the inhibitory effects of both cinnamon and oregano. This blend, if successful in reducing the characteristic flavor of garlic in animal products, could represent a promising CH4 mitigation strategy suitable for practical application. Oregano-based treatments displayed more variable responses. Both (O100) and particularly (O50-C50) caused a marked reduction in GP after day 1, with limited recovery by day 5. This indicates a strongantimicrobial action, likely due to EO active compounds, and points toward potential microbial dysbiosis at higher inclusion levels. Interestingly, the VFA profile of the (O50-C50) blend was more favorable than either oregano or cinnamon used alone, suggesting a complex interaction that may warrant further investigation.

Ternary blends exhibited effects that were abundant-additive dependent, following similar trends observed with each additive alone. The (G33–C33–O33) treatment maintained a moderate and consistent fermentation profile, reaching levels comparable to the control by day 5. Its steady upward trajectory indicates a reduced disturbance of the rumen microbiotaand a more balanced fermentative response. The stable performance further suggests that moderate inclusion levels of cinnamon and oregano are better tolerated under rumen-like conditions. Overall, these results reinforce the idea that

lower doses of essential oils, particularly when incorporated into well-formulated ternary blends, may offer a safer and more effective strategy for modulating rumen fermentation without compromising microbial functionality. In particular, the tertiary combination (G75-O12-C12) resulted in the most promising combination as it decreased CH4 production by 86%, being this effect even greater than reported for the garlic alone (-51%), while maintained GP and VFA similar to the CTL treatment. This strategy could allow decreasing the supply of garlic extract and the subsequent potential negative effects on palatability and smell and taste transfer to the animal products such as milk. As a result, this ternary blend should be investigated further.

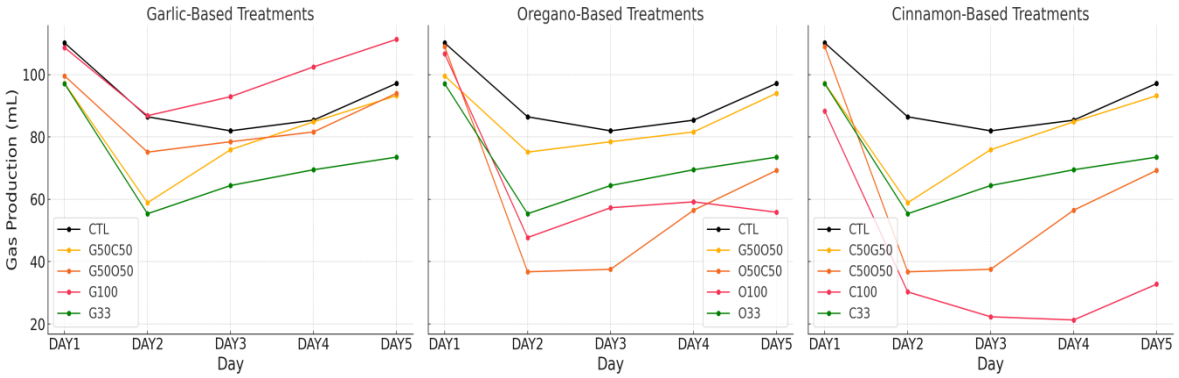


Figure 19: Effects of Single and Combined Additives on Gas Production: Exploring Synergistic and Antagonistic Interactions

Additive, antagonistic and synergistic effects have occurred between components of essential oils (Burt, 2004), suggesting that combinations of essential oils of different composition, or specific combinations of essential oil secondary metabolites, may result in additive and/or synergetic effects which may enhance efficiency of rumen microbial fermentation. Comparison between in vitro and in vivo studies suggests that caution should be taken in extrapolation of*invitro*datato*invivo*conditions. Indeed, many of the concentrations of essential oils that have elicited favorable fermentation responses*invitro*are far too high for*invivo*application (Beauchemin et al., 2009). In our case, most of the combinations showed positive effects on CH_4 reduction and overall fermentation. While the proportions of the different feed additives in the blends could be extrapolated from in vitro to in vivo, we recommend these effects to be evaluated within tolerable dose ranges that do not exert detrimental impacts on animal health or physiological function, as excessive antimicrobial activity may render them unsuitable for on-farm application (Yáñez-Ruiz et al., 2016).

7 CONCLUSIONS

1) Batch culture single incubations and consecutive incubations represent adequate initial approaches to evaluate the mode of action and effectiveness of novel anti-methanogenic feed additives.
2) Dietary supplementation with green seaweeds up to 20% in DM did had minimal effects on the CH_4 emissions and should be discarded as a CH_4mitigation strategy but not as a N source as increased rumen NH_3-N concentration.
3) Garlic extracts demonstrated a strong and consistent antimethanogenic effects, notably reducing CH_4 and NH_3–N concentrations without impairing GP or total VFAsproduction. These effects were mostly due to the presence of diallyl disulfide and other sulfur-containing compounds, which canmodulate the rumen microbial communityand redirect fermentation towards propionate formation as the main hydrogen sink.
4) Dietary supplementation with essential oils such as cinnamon and oregano oils led to a significant decrease in CH_4 production but this was also accompanied by aimportantinhibition of the fermentation when used at high doses.
5) Eugenol supplementationshowed a small anti-microbial ability to decrease CH_4productionwhich was proportional to the inhibition of the fermentation. However, this effect increased over time suggesting the need for microbial adaptation.
6) Polyphenol, as primary sources of gallic acid, led to modest CH_4 reductions but promoted slight improvements in the N metabolism behind this effect dose-dependent. Higher doses should be tested in further experiments.
7) The combinations of different proportion of various additives highlighted that garlic extract provides the dominant anti-methanogenic activity but it could potentially generate palatability or odour transfer issues to the animal products
8) Alternatively, combining garlic extract with other additives such as eugenol and polyphenols (G33-E33-P33) or with oregano and cinnamon (G75-O12-C12) could achieve similar results than obtained with garlic alone but potentially decreasing the negative effects of garlic.
9) While the *in vitro* findings are encouraging, further *in vivo* research is required to confirm these effects under practical conditions, including evaluations of animal performance, palatability, and potential flavor or odortransfer to animal product.

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