INTERNATIONAL DOCTORAL SCHOOL OF THE USC

Ghasaq Sami Mshary

PhD Thesis

ANALYSIS OF FACTORS INFLUENCING GREENHOUSE GAS (GHG) PRODUCTION IN RUMINANTS

Lugo, 2025

Doctoral Programme in Veterinary Medicine and Health

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DOCTORAL THESIS

ANALYSIS OF FACTORS INFLUENCING GREENHOUSE GAS (GHG) PRODUCTION IN RUMINANTS

Ghasaq Sami Mshary

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INTERNATIONAL DOCTORAL SCHOOL OF THE UNIVERSITY OF SANTIAGO OF COMPOSTELA

DOCTORAL PROGRAMME IN VETERINARY MEDICINE AND HEALTH [E2091V01]

LUGO

2025

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CONFLICT OF INTEREST

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DECLARE

that the present PhD Thesis, included in the *Programa de Doutoramento en Medicina e Sanidade Veterinaria* [E2091V01] to which *I acceded by Homologación ou Equivalencia título estranxeiro de fóra do EEES* [50104], does not present any conflict of interest.

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“All tasks can be achieved only
by striving and workin”

Ibn Khaldoon

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ACKNOWLEDGMENTS

At the end of a work as laborious as the development of a doctoral thesis, it is inevitable to concentrate most of the merit on the effort and dedication you have made.

However, from an objective point of view, you are aware that the magnitude of this contribution would not have been possible without the participation of those people who have given their help, their knowledge and their support to carry out this work in the best possible way. It is therefore a real pleasure for me to use this space to express my sincere thanks

First of all, I would like to thank my thesis supervisor Dr. Cristina Castillo Rodriguez, who gave me the opportunity to carry out this doctoral thesis. I am grateful that it allowed me to discover the world of scientific conferences, and to strengthen my scientific training through a doctoral stay at the University of Santiago de Compostela.

I would like to thank in a very special way my director Dr.Muiño Rodrigo who is an example as a professional, but above all as a person. Thanks to their guidance, support and trust, the development of this work has been a real pleasure. Their friendship is without a doubt the best gift I take away from this experience.

In the same way, I could not forget to thank Dr. Joaquin Hernandez who has allowed me to be part of his group Management, providing me with everything I have needed for the development of my professional career.

I thank all of them for the good times shared, moments full of laughter and anecdotes that will go down in history. In addition, I appreciate the encouragement and advice given in the worst moments, as well as dedicating part of your time to help me solve doubts of various kinds. From this experience together, we have continuously learned both personally and professionally from each other and even from ourselves, and for them I thank you.

Within my personal sphere, I am especially grateful to my partner Mohammed Ajmi for having traveled this path of my professional life by my side, throughout which he has given me his unconditional support both for being a wonderful person and for participating in an active and altruistic way in this work. Finally, my deepest and most heartfelt gratitude goes to my daughters Rand, Sarah, Rawan and Noor.

Thanks !

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The current PhD Thesis has been developed thanks to the Research Project entitled ***Study of climate change on health and welfare, reproductive efficiency and milk quality in dairy farms in the countryside of Lugo*** (Galicia, Spain) with the code 2022-PU017 granted by the University of Santiago de Compostela (Campus Terra).

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ABBREVIATIONS (in order of appearance)

IPCC: Intercontinental Panel of Climate Change.
UNFCCC: United Nations Framework Convention on Climate Change.
GHG: Greenhouse gas.
DMI: Dry Matter Intake.
EGF: Epidermal Growth Factor.
SCFA: Short-Chain Fatty Acids.
IGF-1: Insulin-like Growth Factor-1.
VFA: Volatile Fatty Acids.
AA: Amino Acid.
ADF: Acid Detergent Fiber.
NDF: Detergent Detergent Fiber.
ADG: Average Daily Gain.
GEI: Gross Energy Intake.
CP: Crude Protein.
NPN: Non-Protein Nitrogen.
NRC: National Research Council.
RFI: Residual Feed Intake.
EB: Energy Balance.
BW: Body Weight.
ME: Metabolisable Energy.
(3-NOP): 3-nitroxypropanol.
MMIs: Microflora-Modifying Inhibitors.
MON: Monensin.
EIs: Enzymatic Inhibitors.
HRs: Hydrogen Receptors.
BH: Biohydrogenation.
UFA: Unsaturated Fatty Acid.
AGPs: Antibiotic Growth Promoters.
EU: European Union.

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RUP: Rumen Undergradable Protein.

EOs: Essentia Oils.

NASA: National Aeronautics and Space Administration.

NCEI: US National Center for Environmental Information.

IMO: International Maritime Organization.

UN: United Nations.

WMO: World Meteorological Organization.

EPA: Environmental Protection Agency.

PFCs: Perfluorocarbons.

HFCs: Hydrofluorocarbons.

CFCs: Chlorofluorocarbons.

SF6: Sulfur hexafluoride.

NOAA: National Oceanic and Atmospheric Administration.

GWP: Global Warming Potential.

RCPs: Representative Concentration Pathways.

CAPRI: Common Agricultural Policy Regionalized Impact.

CMO: Common Market Organization.

MFA: Milk Fatty Acids.

MY: Milk Yield.

Ym %: Enteric Methane Conversion Factor.

TMR: total mixed ration.

EF: CH_4 emission factor (kg CH_4/head/year).

GE: Gross Energy.

MAPA: Ministry of Agriculture, Fisheries, and Food.

CNCPS: Cornell Net Carbohydrate and Protein System.

MUFA: Monounsaturated Fatty Acids.

PUFA: Polyunsaturated Fatty Acids.

CSPA: Calcium Soap based on Palm Oil Acids.

MCR: Methyl-coenzyme M reductase.

CSPO: Calcium soap based on Palm Oil.

CSFA: Complex of Calcium ions with Long-chain Fatty Acids.

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RESUMO

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RESUMO

O sector vacún de leite en España configúrase como un sector estratéxico, cun nivel de produción de 7.121 millóns de toneladas (supoñendo o 4.5% da produción da Unión Europea, UE). Este sector xoga un rol importante nos medios de vida de centos de miles de familias. Do total de gandeiros de vacún de leite en España, a Cornisa Cantábrica concentrou en 2019 o 79% das mesmos, con Galicia ocupando o 55% da produción leiteira.

Con todo, este sector afronta na actualidade uno dos maiores retos da súa historia máis aló do descenso do número de cabezas e o despoboamento do medio rural. Este reto, en consonancia cos criterios e as estratexias ditadas polo Ministerio de Transición Ecolóxica do Goberno de España, deriva do feito de que devandito grupo está no punto de mira como un dos focos emisores de metano que, xunto cos gases derivados da fermentación das súas excretas, contribúen ao incremento dos Gases de Efecto Invernadoiro (GEI).

Segundo o informe elaborado polo devandito Ministerio, practicamente a metade das emisións deste sector están xeradas polo uso de fertilizantes e a xestión dos chans, mentres que a outra metade está provocada pola gandería (fermentación entérica e xestión de estercos). Entre as actuacións para reducir as emisións de GEI propón o control da fermentación entérica mediante a modificación na dieta para reducir as súas emisións.

En base á información proporcionada e aos extensos datos recompilados por diversos estudos e informes internacionais, a análise precisa dos datos das explotacións de vacún de leite eríxese como unha ferramenta imprescindible na actualidade, permitindo ao profesional veterinario determinar a redución dos factores de emisión de gases de efecto invernadoiro no contexto nacional e internacional.

En consecuencia, o propósito deste estudo é desenvolver un modelo TIER-2 en condicións de campo, utilizando os datos proporcionados polos diversos sistemas de produción de vacún de leite en Galicia, co fin de xerar un inventario de GEI, de acordo con os Estándares Internacionais promovidos polo Panel Internacional para o Cambio Climático (IPCC) e considerando o seu heteroxéneo sistema de produción.

Para alcanzar este obxectivo, exponse os seguintes obxectivos específicos:

1. Determinar os valores de emisión de metano en explotacións lácteas de alta produción asumindo o valor Ym (%) recomendado polo *Refinamento 2019* das Directrices do IPCC de 2006.
2. Avaliar os cambios que presentaban os valores de emisión de metano en función de se os animais atópanse no seu máximo rendemento leiteiro (pico de lactación) ou no período de secado.
3. Explorar o efecto que a dieta fornecida pode ter sobre os resultados de emisión de metano entérico obtidos.

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4. Avaliar, nas condicións da explotación, que compoñentes fisicoquímicos da ración poderían contribuír a reducir as emisións de metano.
5. Avaliar os efectos da suplementación con lípidos da ración sobre os principais compoñentes fisicoquímicos do leite.

Cabe sinalar que, segundo o estado actual dos coñecementos, o presente estudo constitúe a primeira investigación realizada en Galicia no ámbito do vacún de leite que ten en conta as recomendacións do Refinamento 2019 ás Directrices do IPCC de 2006. Esta institución subliña a relevancia de levar a cabo estudos centrados no manexo co fin de realizar análises precisas que permitan establecer modelos preditivos máis refinados como referencia.

Neste sentido, o presente estudo desenvolveuse en instalacións comerciais de vacún de leite que constitúen modelos estándar dentro da industria láctea galega, e non nun centro de investigación, cuxos resultados non sempre poden extrapolarse a condicións de campo.

Neste Proxecto de Tese Doutoral realízase unha primeira valoración relativa ás emisións de metano entérico, empregando métodos indirectos cun potencial de aplicación a gran escala e que foron adquirindo relevancia nos últimos anos. Estes métodos, sobre a base de programas estatísticos sofisticados, permiten a obtención de puntos críticos fiables nos que o especialista poida actuar ao momento. Concretamente recórrese ao emprego do modelo de predición TIER-2 recomendado polo Panel Internacional para o Cambio Climático (IPCC).

Avaliáronse un total de 21 granxas leiteiras da raza Frisoa, situadas na zona da Comarca do Deza (provincia de Pontevedra) e a zona sur da provincia de Lugo. A selección destas explotacións realizouse utilizando como referencia as directrices do IPCC de 2006, establecidas para vacas leiteiras de alta produción, cunha produción anual superior aos 8.500 quilogramos por cabeza. Estas explotacións caracterízanse por proporcionar ás súas animais dietas con máis do 70 % de digestibilidad da calidade do alimento (DE%), segundo a fórmula DE (MJ/kg)/GE (MJ/kg) x100, e unha cantidade inferior ao 35 % de FND (base DMI). En conxunto, o estudo abarca 1530 vacas leiteiras alta produción.

As explotacións seleccionáronse considerando condicións de manexo similares para garantir a aplicabilidade dos datos a este tipo de explotación, baseándose nun sistema de estabulación intensiva en estabulación libre e na formulación da dieta. Os datos solicitados no presente informe foron aprobados polo Comité de Coidado e Uso de Animais da Universidade de Santiago de Compostela, conforme á normativa española (RD 53/2013, disposición legal número 1337) e á normativa europea de animais para fins científicos (Consello de Europa, ETS n.º 123).

A investigación consta de catro apartados ou capítulos secuenciales. En primeiro lugar, no CAPÍTULO 1, realízase unha primeira estimación dos valores de metano entérico (CH_4-EF, kg CH_4/cabeza/ano) nas explotacións de estudo, que se dividen en función da produción de leite e da fase fisiolóxica (pico de lactación e fase de secado). En xeral, os resultados indican que as emisións de metano entérico están significativamente influenciadas pola fase fisiolóxica de lactación e pola composición da dieta, en particular polo IMD e o contido en EE. O grupo con

maior produción de leite emitiu máis metano que o de menor produción, especialmente durante o pico de lactación. A maiores a valoración que fai a IPCC ten en conta todo o ano en lactación, feito que non é real, polo que os datos achegados como referencia poden estar sobredimensionados.

No CAPITULO 2 avalíase o efecto que ten a suplementación con aceite de soia nas emisións de metano entérico en vacas de alta produción leiteira. En concreto, analízanse os perfís de ácidos graxos volátiles (AGV) tanto na forraxe como na ración, valorando a correlación que presentan e como afecta á dinámica ruminal. O estudo demostra o impacto significativo da suplementación con lípidos na dieta sobre as emisións entéricas de metano en vacas leiteiras de alto rendemento.Especificamente, a inclusión de aceite de soia na dieta reduciu significativamente as emisións de metano en comparación cos sales de xabón de calcio. Os resultados suxiren que a composición de ácidos graxos da dieta, en particular a presenza de ácidos graxos específicos como C12:0, C16:1, C18:2, C18:3 (ALA) e ácidos graxos de cadea longa, desempeña un papel fundamental á hora de influír na produción de metano. Estes achados subliñan a relevancia de considerar a composición da dieta nas estratexias deseñadas para mitigar as emisións de gases de efecto invernadoiro provenientes das explotacións leiteiras.

O CAPITULO 3, fai un estudo comparativo entre a suplementación lipídica con aceite de soia e 3 niveis de suplementación con xabóns cálcicos derivados de aceite de palma, valorando ademais o seu efecto sobre a composición físico-química do leite. Os resultados mostraron que o aceite de soia, como suplemento enerxético da ración, aínda que achega menos enerxía bruta á dieta implica unha redución significativa nas emisións de metano entérico respecto a as granxas que reciben a suplementación con xabóns cálcicos, sen que isto afecte aos demais compoñentes da ración. As correlacións realizadas entre as emisións de metano e os valores obtidos en control leiteiro mostraron unha correlación positiva entre estes e os niveis de beta-hidroxibutirato, atribuíbles á menor inxesta enerxética nunha fase de alta demanda metabólica, pero sen que se alcanzasen valores considerados patolóxicos.

Finalmente, o CAPITULO 4 profunda, nun estudo piloto a eficacia dunha alternativa comercial, nitrooxipropanol (o 3-NOP) nun rabaño de alta produción leiteira, considerando os seus efectos sobre as emisións de metano entérico e a calidade do leite. Este suplemento comercial, efectivamente pode ser un sustitutivo aos suplementos de orixe natural, xa que chega a reducir ata un 25% as emisións de metano. Con todo, a redución na produción de leite, así como de certos compoñentes do leite, recomendan seguir profundando no seu estudo en distintas condicións de manexo e en busca da dieta que se adapta á súa maior eficacia.

Todos estes estudos experimentais, e sobre a base dos obxectivos expostos, lévannos a concluír que

1. De acordo con o modelo TIER-2, recomendado polo *2019 Refinement 2006 IPCC Guidelines*, a emisión de metano entérico (CH_4-EF, kg CH_4/cabeza/ano) das vacas leiteiras vese significativamente influenciada por diversos factores. Devanditos factores

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inclúen a inxesta de materia seca, o estadio fisiolóxico e a composición da dieta, especialmente o contido de graxa.

2. As vacas de alta produción emiten maiores cantidades de metano, especialmente durante o pico de lactación. Os factores de emisión de metano (CH_4-EF, kg CH_4/cabeza/ano), definidos seguindo o modelo TIER-2, céntranse na fase de lactación e estendéronse ata abarcar todo o ciclo produtivo. Con todo, é importante sinalar que estes datos non reflicten con precisión a realidade gandeira. Por tanto, é necesario recalibrar a avaliación da emisión de metano segundo a fase de lactación. De acordo con este enfoque, os valores de emisión de metano entérico dos animais estudados son inferiores aos establecidos para os países desenvolvidos. Este achado expón interrogantes sobre o papel do sector bovino no contexto do cambio climático e a necesidade de buscar medidas para minimizar as emisións de metano. Con todo, desde unha perspectiva produtiva, a síntese de metano conleva un custo enerxético para o metabolismo do animal, o que podería afectar outras funcións produtivas
3. O tipo de suplemento graxo determina as variacións nas emisións entéricas de metano (CH_4-EF, kg CH_4/cabeza/ano). O uso de suplementos lipídicos, como o aceite de soia, considérase unha estratexia efectiva para mitigar as emisións de metano, xa que estas fontes considéranse «naturais» e son valoradas tanto desde o punto de vista ambiental como polos consumidores. Observouse que o aceite de soia, cando se inclúe na dieta, reduce significativamente as emisións de metano en comparación cos sales de xabón de calcio, independentemente da doce.
4. Considerando a suplementación lipídica administrada tradicionalmente nas explotacións da zona de estudo, a suplementación con aceite de soia é preferible á suplementación con xabón de calcio, sen afectar negativamente á calidade fisicoquímica do leite producido (proteína, graxa, ácidos graxos poliinsaturados ou monoinsaturados). Observouse unha correlación positiva cos niveis de corpos cetónicos (beta-hidroxibutirato), probablemente debido á menor achega enerxética do aceite de soia. Esta correlación podería servir como biomarcador temperán que indique que a dieta administrada ten potencial metanogénico.
5. Na procura de novas fontes de graxa suplementaria para minimizar as emisións de metano entérico, o uso de nitrooxipropanol (3-NOP) como substituto do aceite de soia reduce ao redor dun 25% as emisións de metano entérico en comparación coa suplementación con aceites de xabón de calcio, o suplemento tradicional e comunmente utilizado no gando leiteiro de alta produción. Con todo, este suplemento, nas condicións do noso estudo, provocou unha lixeira diminución da produción de leite, graxa e proteína, atribuíble á composición da dieta e ao manexo da explotación, aínda que non na mesma magnitude que as emisións entéricas de metano.

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RESUMEN

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RESUMEN

El sector vacuno de leche en España se configura como un sector estratégico, con un nivel de producción de 7.121 millones de toneladas (suponiendo el 4.5% de la producción de la Unión Europea, UE). Este sector juega un rol importante en los medios de vida de cientos de miles de familias. Del total de ganaderos de vacuno de leche en España, la Cornisa Cantábrica concentró en 2019 el 79% de las mismos, con Galicia ocupando el 55% de la producción lechera.

Sin embargo, este sector afronta en la actualidad uno de los mayores retos de su historia más allá del descenso del número de cabezas y el despoblamiento del medio rural. Este reto, en consonancia con los criterios y las estrategias dictadas por el Ministerio de Transición Ecológica del Gobierno de España, deriva del hecho de que dicho grupo está en el punto de mira como uno de los focos emisores de metano que, junto con los gases derivados de la fermentación de sus excretas, contribuyen al incremento de los Gases de Efecto Invernadero (GEI).

Según el informe elaborado por dicho Ministerio, prácticamente la mitad de las emisiones de este sector están generadas por el uso de fertilizantes y la gestión de los suelos, mientras que la otra mitad está provocada por la ganadería (fermentación entérica y gestión de estiércoles). Entre las actuaciones para reducir las emisiones de GEI propone el control de la fermentación entérica mediante la modificación en la dieta para reducir sus emisiones.

En base a la información proporcionada y a los extensos datos recopilados por diversos estudios e informes internacionales, el análisis preciso de los datos de las explotaciones de vacuno de leche se erige como una herramienta imprescindible en la actualidad, permitiendo al profesional veterinario determinar la reducción de los factores de emisión de gases de efecto invernadero en el contexto nacional e internacional.

En consecuencia, el propósito de este estudio es desarrollar un modelo TIER-2 en condiciones de campo, utilizando los datos proporcionados por los diversos sistemas de producción de vacuno de leche en Galicia, con el fin de generar un inventario de GEI, de acuerdo con los Estándares Internacionales y considerando su heterogéneo sistema de producción.

Para alcanzar este objetivo, se plantean los siguientes objetivos específicos:

1. Determinar los valores de emisión de metano en explotaciones lácteas de alta producción asumiendo el valor Ym (%) recomendado por el *Refinamiento 2019 de las Directrices del IPCC de 2006*.
2. Evaluar los cambios que presentaban los valores de emisión de metano en función de si los animales se encuentran en su máximo rendimiento lechero (pico de lactación) o en el periodo de secado.
3. Explorar el efecto que la dieta suministrada puede tener sobre los resultados de emisión de metano entérico obtenidos.
4. Evaluar, en las condiciones de la explotación, qué componentes fisicoquímicos de la ración podrían contribuir a reducir las emisiones de metano.
5. Evaluar los efectos de la suplementación con lípidos de la ración sobre los principales componentes fisicoquímicos de la leche.

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Cabe señalar que, según el estado actual de los conocimientos, el presente estudio constituye la primera investigación realizada en Galicia en el ámbito del vacuno de leche que tiene en cuenta las recomendaciones del Refinamiento 2019 a las Directrices del IPCC de 2006. Esta institución subraya la relevancia de llevar a cabo estudios centrados en el manejo con el fin de realizar análisis precisos que permitan establecer modelos predictivos más refinados como referencia.

En este sentido, el presente estudio se ha desarrollado en instalaciones comerciales de vacuno de leche que constituyen modelos estándar dentro de la industria láctea gallega, y no en un centro de investigación, cuyos resultados no siempre pueden extrapolarse a condiciones de campo.

En este Proyecto de Tesis Doctoral se realiza una primera valoración relativa a las emisiones de metano entérico, empleando métodos indirectos con un potencial de aplicación a gran escala y que han ido adquiriendo relevancia en los últimos años. Estos métodos, sobre la base de programas estadísticos sofisticados, permiten la obtención de puntos críticos fiables en los que el especialista pueda actuar al momento. Concretamente se recurre al empleo del modelo de predicción TIER-2 recomendado por el Panel Internacional para el Cambio Climático (IPCC).

Se evaluaron un total de 21 granjas lecheras de la raza Frisona, ubicadas en la zona de la Comarca del Deza (provincia de Pontevedra) y la zona sur de la provincia de Lugo. La selección de estas explotaciones se realizó utilizando como referencia las directrices del IPCC de 2006, establecidas para vacas lecheras de alta producción, con una producción anual superior a los 8.500 kilogramos por cabeza. Estas explotaciones se caracterizan por proporcionar a sus animales dietas con más del 70 % de digestibilidad de la calidad del alimento (DE%), según la fórmula DE (MJ/kg)/GE (MJ/kg) x100, y una cantidad inferior al 35 % de FND (base DMI). En conjunto, el estudio abarca 1530 vacas lecheras alta producción.

Las explotaciones se seleccionaron considerando condiciones de manejo similares para garantizar la aplicabilidad de los datos a este tipo de explotación, basándose en un sistema de estabulación intensiva en estabulación libre y en la formulación de la dieta. Los datos recabados en el presente informe fueron aprobados por el Comité de Cuidado y Uso de Animales de la Universidad de Santiago de Compostela, conforme a la normativa española (RD 53/2013, disposición legal número 1337) y a la normativa europea de animales para fines científicos (Consejo de Europa, ETS n.º 123).

La investigación consta de cuatro apartados o capítulos secuenciales. En primer lugar, en el CAPÍTULO 1, se realiza una primera estimación de los valores de metano entérico (CH_4-EF, kg CH_4/cabeza/año) en las explotaciones de estudio, que se dividen en función de la producción de leche y de la fase fisiológica (pico de lactación y fase de secado). En general, los resultados indican que las emisiones de metano entérico están significativamente influenciadas por la fase fisiológica de lactación y por la composición de la dieta, en particular por el IMD y el contenido en EE. El grupo con mayor producción de leche emitió más metano que el de menor producción, especialmente durante el pico de lactación. A mayores la valoración que hace la IPCC tiene en cuenta todo el año en lactación, hecho que no es real, por lo que los datos aportados como referencia pueden estar sobredimensionados.

En el CAPITULO 2 se evalúa el efecto que tiene la suplementación con aceite de soja en las emisiones de metano entérico en vacas de alta producción lechera. En concreto, se analizan los perfiles de ácidos grasos volátiles (AGV) tanto en el forraje como en la ración, valorando la correlación que presentan y como afecta a la dinámica ruminal. El estudio demuestra el impacto

significativo de la suplementación con lípidos en la dieta sobre las emisiones entéricas de metano en vacas lecheras de alto rendimiento. Específicamente, la inclusión de aceite de soja en la dieta redujo significativamente las emisiones de metano en comparación con las sales de jabón de calcio. Los resultados sugieren que la composición de ácidos grasos de la dieta, en particular la presencia de ácidos grasos específicos como C12:0, C16:1, C18:2, C18:3 (ALA) y ácidos grasos de cadena larga, desempeña un papel fundamental a la hora de influir en la producción de metano. Estos hallazgos subrayan la relevancia de considerar la composición de la dieta en las estrategias diseñadas para mitigar las emisiones de gases de efecto invernadero provenientes de las explotaciones lecheras.

El CAPITULO 3, hace un estudio comparativo entre la suplementación lipídica con aceite de soja y 3 niveles de suplementación con jabones cálcicos derivados de aceite de palma, valorando además su efecto sobre la composición físico-química de la leche. Los resultados mostraron que el aceite de soja, como suplemento energético de la ración, si bien aporta menos energía bruta a la dieta implica una reducción significativa en las emisiones de metano entérico respecto a las granjas que reciben la suplementación con jabones cálcicos, sin que esto afecte a los demás componentes de la ración. Las correlaciones realizadas entre las emisiones de metano y los valores obtenidos en control lechero mostraron una correlación positiva entre éstos y los niveles de beta-hidroxibutirato, atribuibles a la menor ingesta energética en una fase de alta demanda metabólica, pero sin que se alcanzaran valores considerados patológicos.

Finalmente, el CAPITULO 4 ahonda, en un estudio piloto la eficacia de una alternativa comercial, el 3-NOP en un rebaño de alta producción lechera, considerando sus efectos sobre las emisiones de metano entérico y la calidad de la leche. Este suplemento comercial, efectivamente puede ser un sustitutivo a los suplementos de origen natural, ya que llega a reducir hasta un 25% las emisiones de metano. No obstante, la reducción en la producción de leche, así como de ciertos componentes de la leche, recomiendan seguir profundizando en su estudio en distintas condiciones de manejo y en busca de la dieta que se adapta a su mayor eficacia.

Todos estos estudios experimentales, y sobre la base de los objetivos planteados, nos llevan a concluir que

1. De acuerdo con el modelo TIER-2, recomendado por el *2019 Refinement to the 2006 IPCC Guidelines*, la emisión de metano entérico (CH_4-EF, kg CH_4/cabeza/año) de las vacas lecheras se ve significativamente influenciada por diversos factores. Dichos factores incluyen la ingesta de materia seca, el estadio fisiológico y la composición de la dieta, especialmente el contenido de grasa.
2. Las vacas de alta producción emiten mayores cantidades de metano, especialmente durante el pico de lactación. Los factores de emisión de metano (CH_4-EF, kg CH_4/cabeza/año), definidos siguiendo el modelo TIER-2, se centran en la fase de lactación y se han extendido hasta abarcar todo el ciclo productivo. Sin embargo, es importante señalar que estos datos no reflejan con precisión la realidad ganadera. Por lo tanto, es necesario recalibrar la evaluación de la emisión de metano según la fase de lactación, como se ha sugerido en el informe del IPCC. De acuerdo con este enfoque, los valores de emisión de metano entérico de los animales estudiados son inferiores a los establecidos para los países desarrollados. Este hallazgo plantea interrogantes sobre el papel del sector bovino en el contexto del cambio climático y la necesidad de buscar medidas para minimizar las emisiones de metano. Sin embargo, desde una perspectiva

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productiva, la síntesis de metano conlleva un costo energético para el metabolismo del animal, lo que podría afectar otras funciones productivas.

3. El tipo de suplemento graso determina las variaciones en las emisiones entéricas de metano (CH_4-EF, kg CH_4/cabeza/año). El uso de suplementos lipídicos, como el aceite de soja, se considera una estrategia efectiva para mitigar las emisiones de metano, ya que estas fuentes se consideran «naturales» y son valoradas tanto desde el punto de vista medioambiental como por los consumidores. Se ha observado que el aceite de soja, cuando se incluye en la dieta, reduce significativamente las emisiones de metano en comparación con las sales de jabón de calcio, independientemente de la dosis
4. Considerando la suplementación lipídica administrada tradicionalmente en las explotaciones de la zona de estudio, la suplementación con aceite de soja es preferible a la suplementación con jabón de calcio, sin afectar negativamente a la calidad fisicoquímica de la leche producida (proteína, grasa, ácidos grasos poliinsaturados o monoinsaturados). Se ha observado una correlación positiva con los niveles de cuerpos cetónicos (beta-hidroxibutirato), probablemente debido al menor aporte energético del aceite de soja. Esta correlación podría servir como biomarcador temprano que indique que la dieta administrada tiene potencial metanogénico.
5. En la búsqueda de nuevas fuentes de grasa suplementaria para minimizar las emisiones de metano entérico, el uso de nitrooxipropanol (3-NOP) como sustituto del aceite de soja reduce alrededor de un 25% las emisiones de metano entérico en comparación con la suplementación con aceites de jabón de calcio, el suplemento tradicional y comúnmente utilizado en el ganado lechero de alta producción. No obstante, este suplemento, en las condiciones de nuestro estudio, provocó una ligera disminución de la producción de leche, grasa y proteína, atribuible a la composición de la dieta y al manejo de la explotación, aunque no en la misma magnitud que las emisiones entéricas de metano.

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SUMMARY

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SUMMARY

The dairy cattle sector in Spain is a strategic sector, with a production level of 7.121 million tons (accounting for 4.5% of the production of the European Union, EU). This sector plays an important role in the livelihoods of hundreds of thousands of families. In 2019, the Cantabrian coast was home to 79% of all dairy cattle farmers in Spain, with Galicia accounting for 55% of milk production.

However, the sector is currently facing significant challenges, including a decline in the number of heads and depopulation of rural areas. This challenge aligns with the criteria and strategies outlined by the Ministry of Ecological Transition of the Spanish Government, as the sector is a focal point for methane emissions. These emissions, derived from the fermentation of excreta, contribute to the increase of Greenhouse Gases (GHG).

According to the report compiled by the Ministry, approximately 50% of emissions in this sector are attributable to fertilizers and soil management, with the remaining 50% stemming from livestock (enteric fermentation and manure management). Among the proposed measures to mitigate greenhouse gas (GHG) emissions are strategies to control enteric fermentation through dietary modification, to reduce emissions.

Based on the information provided and the wide data registered by different studies or international reports, precise data analysis from dairy cattle farms is an essential tool today that will allow the veterinarian to decide to reduce the Greenhouse Gas Emissions Factors within the national/international context.

Therefore, the main objective of the study is to develop a TIER-2 model at on-farm conditions based on the data provided by the different dairy production systems in Galicia to create a GHG inventory, within the framework of the International Standards and considering its varied production system.

To achieve this objective, the following specific objectives are set out:

1. To determine methane emission values in high-production dairy farms assuming the Ym value (%) recommended by the *2019 Refinement to the 2006 IPCC Guidelines*.
2. To evaluate the changes that EF showed depending on whether the animals are at their maximum milk yield (peak lactation) or in the dry-off period.
3. To explore the effect that the diet supplied may have on the CH_4-EF results achieved.
4. To evaluate, at on-farm conditions, which physicochemical components of the ration could contribute to lower emissions of methane.
5. To assess the effects of lipid supplementation on the main physicochemical components of milk

We would furthermore like to point out that to the best of our knowledge, this is the first study carried out in Galicia on dairy cattle that considers the recommendations of the 2019 Refinement to the 2006 IPCC Guidelines. This institution emphasizes the importance of conducting studies that focus on management to carry out precise analyses for establishing more refined predictive models as a reference.

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With this purpose, this research was carried out in commercial dairy facilities that are standard models within the Galician dairy industry and not in a research center whose results cannot always be extrapolated to field conditions.

The farms were selected considering similar management conditions to ensure applicable data for this type of exploitation and based on an intensive free-stall housing system and diet formulation. The collected data conducted in this report was approved by the Animal Care and Use Committee of the University of Santiago de Compostela, according to the Spanish Regulations (RD 53/2013, legal provision number 1337) and the European regulation of animals for scientific purposes (Council of Europe, ETS no.123).

This Doctoral Thesis Project assesses enteric methane emissions using indirect methods with the potential for large-scale application. These methods, based on sophisticated statistical programs, provide reliable critical points on which specialists can act immediately. Specifically, the TIER-2 prediction model recommended by the International Panel on Climate Change (IPCC) is used.

A total of 21 dairy farms of the Friesian breed, located in the Deza region (province of Pontevedra) and the southern area of the province of Lugo, were evaluated. The selection of these farms was made using as a reference the 2006 IPCC guidelines, established for high-production dairy cows, with an annual production of more than 8.500 kilograms per head. These farms are distinguished by their commitment to providing animals with diets characterized by over 70% feed quality digestibility (DE%), as calculated using the formula DE (MJ/kg)/GE (MJ/kg) x100, and less than 35% NDF (DMI basis). The study encompasses a total of 1.530 high-producing dairy cows.

The research is divided into four sequential sections or chapters. First, in CHAPTER 1, an initial estimate of enteric methane values (CH_4-EF, kg CH_4/head/year) is made for the study farms, which are divided according to milk production and physiological phase (lactation peak and drying phase). The results indicate that enteric methane emissions are significantly influenced by the physiological phase of lactation and by the diet composition, in particular by the IMD and EE content. The IPCC assessment takes into account the entire lactation year, which is not realistic, so the data provided as a reference may be overestimated.

CHAPTER 2 evaluates the effect of soybean oil supplementation on enteric methane emissions in high-producing dairy cows. Specifically, volatile fatty acid (VFA) profiles in both forage and ration are analyzed, assessing their correlation and how they affect rumen dynamics. The study demonstrates the significant impact of dietary lipid supplementation on enteric methane emissions in high-yielding dairy cows. Specifically, including soybean oil in the diet significantly reduced methane emissions compared to calcium soap salts. The results suggest that the fatty acid composition of the diet, in particular the presence of specific fatty acids such as C12:0, C16:1, C18:2, C18:3 (ALA), and long-chain fatty acids, plays a key role in influencing methane production. These findings underscore the relevance of considering diet composition in strategies designed to mitigate greenhouse gas emissions from dairy farms.

CHAPTER 3, makes a comparative study between lipid supplementation with soybean oil and 3 levels of supplementation with calcium soaps derived from palm oil, also evaluating its effect on the physicochemical composition of the milk. The results showed that soybean oil, as an energy supplement to the ration, although it contributes less gross energy to the diet, implies a significant reduction in enteric methane emissions concerning the farms that receive

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supplementation with calcium soaps, without affecting the other components of the ration. The correlations made between methane emissions and the values obtained in dairy control showed a positive correlation between these and beta-hydroxybutyrate levels, attributable to the lower energy intake in a phase of high metabolic demand, but without reaching values considered pathological.

Finally, CHAPTER 4 delves, in a pilot study, into the efficacy of a commercial alternative, 3-NOP, in a high milk production herd, considering its effects on enteric methane emissions and milk quality. This commercial supplement can indeed be a substitute for naturally occurring supplements, as it can reduce methane emissions by up to 25%. However, the reduction in milk production, as well as in certain components of milk, recommends further study under different conditions of production.

All these experimental studies, and based on the stated objectives, lead us to conclude that

1. According to the TIER-2 model, recommended by the 2019 Refinement to the 2006 IPCC Guidelines, enteric methane emission (CH_4-EF, kg CH4/head/year) of dairy cows is significantly influenced by several factors. These factors include dry matter intake, physiological stage and diet composition, especially fat content.

2. High-producing cows emit higher amounts of methane, especially during peak lactation. Methane emission factors (CH_4-EF, kg CH_4/head/year), defined following the TIER-2 model, focus on the lactation phase and have been extended to cover the entire production cycle. However, it is important to note that these data do not accurately reflect the reality of livestock production. Therefore, it is necessary to recalibrate the assessment of methane emissions according to the lactation phase, as suggested in the IPCC report. According to this approach, the enteric methane emission values of the animals studied are lower than those established for developed countries. This finding raises questions about the role of the cattle sector in the context of climate change and the need to seek measures to minimize methane emissions. However, from a productive perspective, methane synthesis entails an energy cost for the animal's metabolism, which could affect other productive functions.

3. The type of fat supplement determines variations in enteric methane emissions (CH_4-EF, kg CH_4/head/year). The use of lipid supplements, such as soybean oil, is considered an effective strategy to mitigate methane emissions, as these sources are considered “natural” and are valued both environmentally and by consumers. Soybean oil, when included in the diet, has been observed to significantly reduce methane emissions compared to calcium soap salts, regardless of the dose

4. Considering the lipid supplementation traditionally administered on farms in the study area, supplementation with soybean oil is preferable to supplementation with calcium soap, without negatively affecting the physicochemical quality of the milk produced (protein, fat, polyunsaturated or monounsaturated fatty acids). A positive correlation has been observed with the levels of ketone bodies (beta-hydroxybutyrate), probably due to the lower energy intake of soybean oil. This correlation could serve as an early biomarker indicating that the diet administered has methanogenic potential.

5. In the search for new sources of supplemental fat to minimize enteric methane emissions, the use of nitrooxypropanol (3-NOP) as a soybean oil substitute reduces enteric methane emissions by about 25% compared to supplementation with calcium soap oils, the traditional and

commonly used supplement in high-producing dairy cattle. However, this supplementation, under the conditions of our study, caused a slight decrease in milk, fat, and protein production, attributable to diet composition and farm management, although not to the same magnitude as enteric methane emissions.

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INTRODUCTION

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JUSTIFICATION OF THE TOPIC.

Growing doubts regarding the significant rise in greenhouse gas emissions have surfaced during the last two decades. According to the *Intercontinental Panel of Climate Chang* (IPCC, 2023) and Patra and Saxena (2010), studies show some gases such as CH_4, NO_2, and CO_2 have a detrimental effect on the ecosystem and contribute to global warming, which causes climate change and environmental degradation. These gas emissions have an impact on productivity in addition to the environment (Giger-Reverdin and Sauvant, 2000). 31% of the world's methane emissions come from agriculture, and 78% of that comes from enteric fermentation, principally from the rumen of cows (UNFCCC, 2015).

Despite CH_4 having a warming potential 21 times greater than CO_2, it may contribute even more to GHG emissions **(Figure 1)**. In contrast to other greenhouse gases, which have an atmospheric lifespan of 120 years (for CO_2 and 114 years for N_2O), CH_4 has a short lifetime of 10–12 years (Sejian and Naqvi, 2012). Because CH_4 exists in the stomachs, the primary way that ruminants emit it is by eructation. It is well-established that the kind and size of the animal, the amount of feed they consume, and the meal's digestibility all affect how much CH_4 an animal can release (Wilkerson *et al*., 1995).

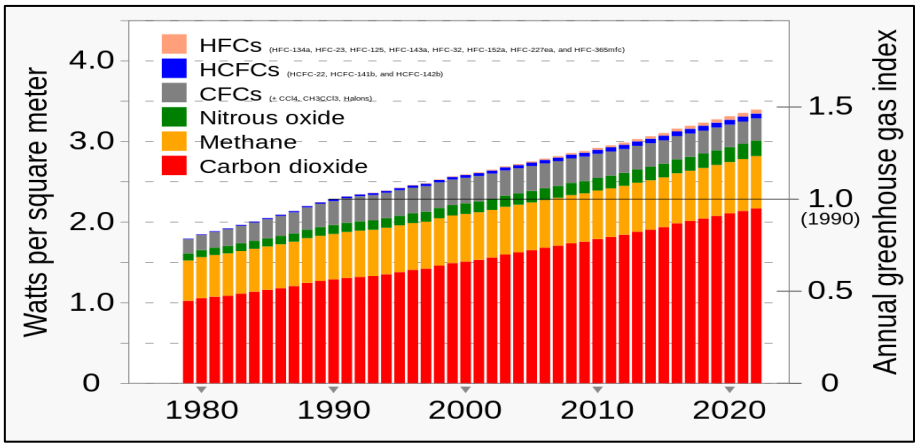

Figure 1. The warming effect of greenhouse gases (GHG_s) (https://gml.noaa.gov/aggi/aggi.html – Archive thereof: https://web.archive.org/web/2023).

According to Orskov *et al.* (1968), Monteny *et al*. (2001), and Kebreab *et al*. (2006), methane is the crucial reaction product of anaerobic bacterial fermentation from CO_2 and H_2 generated from the fermentation of carbon sources, essentially structural and storage carbohydrates in plants. Methane generation is generally the result of microbial fermentation of hydrolyzed carbohydrates and represents an energy loss for the host (Kristensen *et al*., 2011; Getabalew *et al*., 2020). One of the by-products is methane, which has the unique property of being poorly soluble in water it volatilizes from liquids with ease (**Figure 2**).

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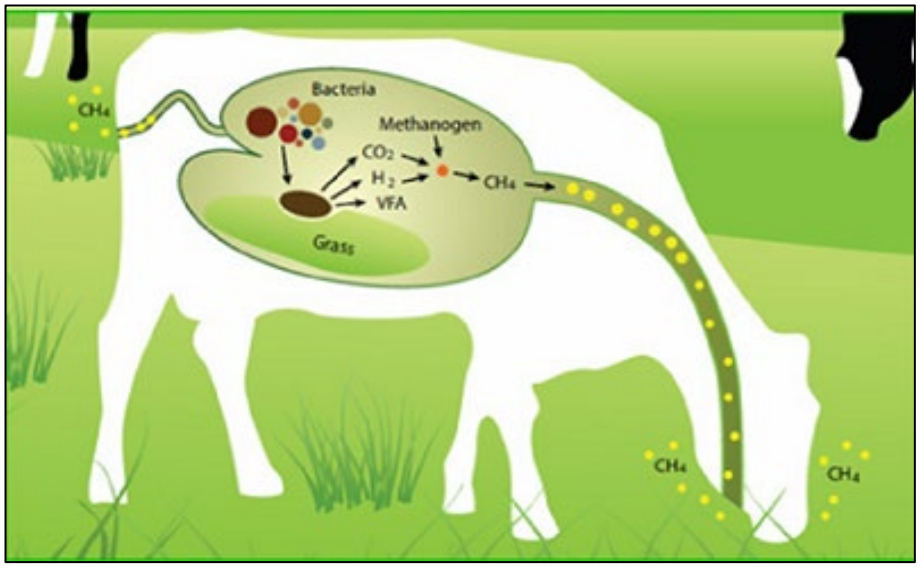

Figure 2. An overview of the methane emissions process (Agricultural-Emissions---greenhouse-gases-and-ammonia-factsheet.pdf (teagasc.ie).

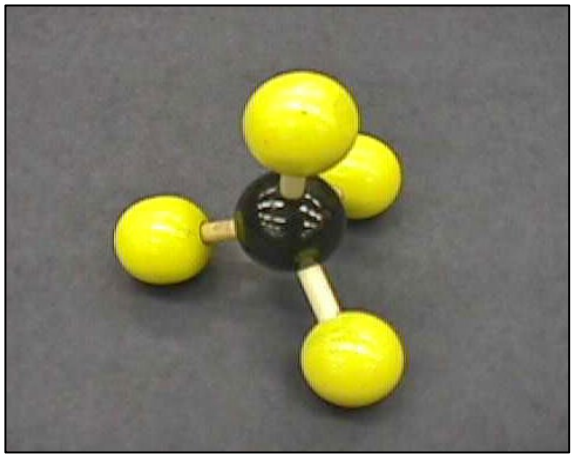

Figure 3. A chemical "ball and stick" model illustrates how a methane molecule is made up of one carbon atom encircled by four hydrogen atoms. Adapted from (Schmidt, 2006).

The two elements that constitute methane's (CH_4) substance are carbon and hydrogen (**Figure 3**). There are two stable isotopes for each element: protium (1H) and deuterium (2H or D); they are carbon-12 (12C) and carbon-13 (13C), respectively (Monteny *et al*., 2001). Methane's carbon isotopic structure enables differentiation between its specific sources. Microbial methane is highly depleted in 13C, whereas thermogenic methane, specifically, is emitted during oil and gas production and tends to be rich (López *et al*., 2017). Methanogens are specific kinds of microbes that are the only ones that can create methane. Substrate fermentation and metabolism have accounted for the variation in methanogenic activity between various gut regions and animal species. Because cattle have larger bodies and more heads than other ruminant species, they are the primary source of this intestinal methane. Methanogens primarily use three substrates: CO_2, acetate, and molecules with methyl groups

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(De la Fuente *et al*., 2019). A previous study (Johnson and Johnson, 1995) showed animals' daily emissions of CH_4 varied from 250 to 500 L per animal. Ruminants create methane during digestion, thus contributing to the greenhouse effect and waste feed energy (Liu *et al*., 2022). Calves that consume more organic matter have larger rumen physiological capacities, longer retention durations, and higher digestion rates, which increase CH_4 generation (Pinares-Patiño *et al*., 2016; Huhtanen *et al*., 2016). Enteric CH_4 can be expressed in a variety of ways, including as the total amount produced per day (i.e., total CH_4 production), as a percentage of the gross energy ingested (i.e., CH_4 conversion factor, Ym%), as a product such as meat or milk (i.e., CH_4 intensity), or as an absolute amount emitted per day (i.e., total CH_4 yield, Vargas *et al*., 2022).

The amount of intake, the kind and quality of feeds, energy intake, animal size, growth rate, production level, genetics, and ambient temperature are a few of the many variables that affect ruminant CH_4 output (Hegarty, 2004). In addition to producing one of the most significant greenhouse gases, (**Figure 2**) their function in maintaining the equilibrium of hydrogen pressure is critical to the functioning of the rumen ecosystem (Huws *et al*., 2018).

Prior research (Jiao *et al*., 2014; Fu *et al.,* 2022) on the impact of methane generation in ruminants, such as cattle and deer, suggested that age was likely connected to animal parameters such as body weight, dry matter intake (DMI), and retention time (Moss *et al*., 2000). Simultaneously, rumen microbiome alterations are related to age and physiological status (Kumar *et al*., 2015; Pitta *et al*., 2016a; Dill-McFarland *et al*., 2017).

Yet, increasing meat and milk production contributes to raising GHG. According to estimates, cattle production accounts for 9% to 11% of all anthropogenic greenhouse gas emissions worldwide (Pickering *et al*., 2015), with methane accounting for about 44% of cattle emissions of CH_4 (Rojas-Downing *et al*., 2017). The most crucial factors are the type and quality of forage and the level of additives in the concentrate or forage.

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1. PHYSIOLOGICAL AND ANATOMICAL DESCRIPTION OF THE DIGESTIVE TRACT OF RUMINANTS

The ruminant digestive system's physiology and structure developed billions of years ago to facilitate the efficient digestion of different polysaccharides and cellulose materials (Oltjen and Beckett, 1996). The digestive sac of a ruminant animal consists of four compartments: the reticulum, omasum, abomasum, and rumen. The reticulorumen fold divides the rumen's internal environment into distinct sacs, with the reticulum being the first sac to receive food after it passes through the rumen (**Figure 4**). According to Shibata and Terada. (2010), the rumen contains 15 liters for sheep and 100 to 150 liters for cattle, accounting for more than 70% of the stomach's total capacity. As a fermentative vat that transforms plant matter into nutrients the host might require, the rumen is a large, glandular foregut compartment. The esophageal canal allows ingested feed to pass through the rumen, exposing it to a dense, multidomain microbial community (Dehority, 2003). The system's design, which allows the swallowed feed material to interact with microflora for an extended period, gives them effectiveness (Krause *et al*., 2003).

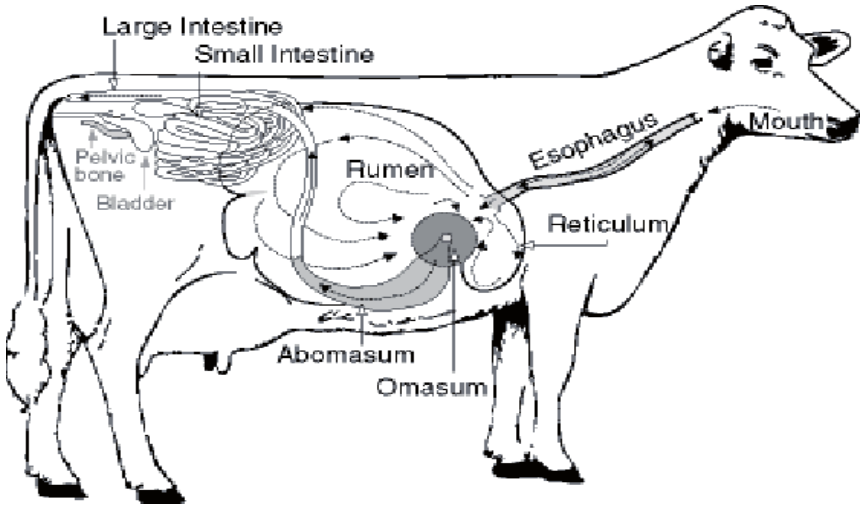

Figure 4. Cow's digestive tract sections. Adapted from (Böhnlein, 2007).

Meal intake flows from the reticulum to the rumen (Castillo *et al*., 2014). The stratified squamous epithelium lining the rumen has papillae that resemble leaves. The feed is ingested and stored in the reticulorumen (collective chamber of reticulum and rumen) for ruminant feeding and interaction with microorganisms. After the chewing process, the food is swallowed. Following that, the feed moves to the anterior reticulorumen. Both ingestion and rumination depend on saliva. The acids produced during bacterial fermentation also have high quantities of potassium, sodium bicarbonate, and phosphate (Owens and Basalan, 2016).

According to Sahu *et al*. (2002), the reticulorumen appears to be a multipurpose fermentation sac that ranges in size from 35 to 100 L for cattle. The environment in the reticulorumen is primarily anaerobic, with carbon dioxide (65%), methane (27%), nitrogen (7%), and hydrogen (0.2%) (Burns, 2008; Kingston-Smith *et al*., 2013). In addition to these,

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there are also trace amounts of CO, H_2S, and O_2. The rumen's extensive fermentation by the local microbiota is the mechanism of gas composition. Plants that contain complex polysaccharides, such as hemicellulose, cellulose, and lignin, are the primary food for ruminants (Flint and Bayer, 2008). But instead of using an enzymatic system to break down polysaccharides, they use gut microflora that hydrolyzes these substances to produce energy (Flint *et al*., 2008).

As a result, forage consumption triggers fermentation, which matures the rumen function in response to the release of VFA, and hormones like insulin, insulin-like growth factor IGF-1, and epidermal growth factor (EGF) (**Figure 5**) (Öztürk and Gursel, 2021; Fu et al., 2022). These commensal microorganisms have a synergistic connection with the host by using the nutrients that the host consumes for survival. The reticulorumen compartment serves as a habitat for the microbial population. The reticulorumen is a place of dense and varied microflora that includes eukaryotes (fungi and protozoa), archaea (methanogens), and bacteria (Ozutsumi *et al*., 2005). These bacterial communities appear highly sensitive, responding to even little alterations in the rumen environment (**Figure 6**).

The rumen microflora's fermentation process is multifaceted in that microbes collaborate to produce simpler chemicals that the host can readily metabolize (Mcallister *et al*., 1990). Herbivore digestive physiology is conceptualized as the problem of maximizing feed intake, diet quality, and digestibility (Hume, 2005). The level of feed intake is typically negatively related to digesta mean retention time (Müller *et al.,* 2013).

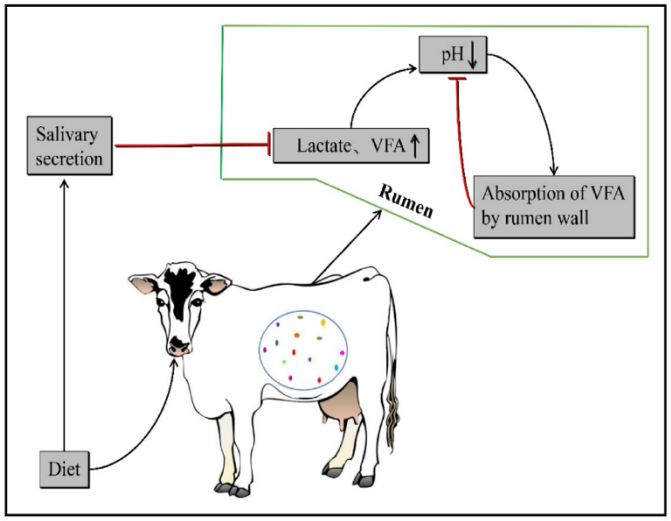

Figure 5. The mechanism of fermentation in the rumen of dairy cows. Adapted from (Fu *et al*., 2022).

The intake-retention time relationship can be modulated by gut capacity and particle size reductions in chewing efficiency (Clauss *et al*., 2007; 2009d). The hallmark of ruminant digestive physiology is the remarkable reduction in ingesta particle size that functional ruminants achieve (Fritz *et al*., 2009). This reduction enables them to increase intake without compromising digestibility (as compared to non-ruminant foregut fermenters) (Schwarm *et al*., 2009; Clauss *et al*., 2015). Furthermore, with most microbial cells in the growth and reproductive stages, the metabolic state of the ruminal microflora is probably geared towards quicker growth rates, possibly fueled by a higher digesting capacity of the microorganisms (Isaacson *et al*., 1975; Hummel *et al*., 2015). A decline in methane could also result from this

change in microbial metabolism (Isaacson *et al*., 1975; Van Nevel and Demeyer, 1979; Shi *et al*., 2014). This alteration is a consequence of something akin to the "partitioning factor" of feeds or the extent to which they initiate energy transfer into the creation of short-chain fatty acids or microbes and, consequently, CH_4 (Clauss and Hummel, 2017). Increased salivation should also result in a higher fluid flow, which will protect against acidosis. It is possible to breed ruminants with different average digesta retention times (Smuts *et al*., 1995; Goopy *et al*., 2014). Pinares-Patiño *et al*. (2013) suggest that the most conceivable reason for the genetic differences in methane emissions is a ruminal digesta retention time variable.

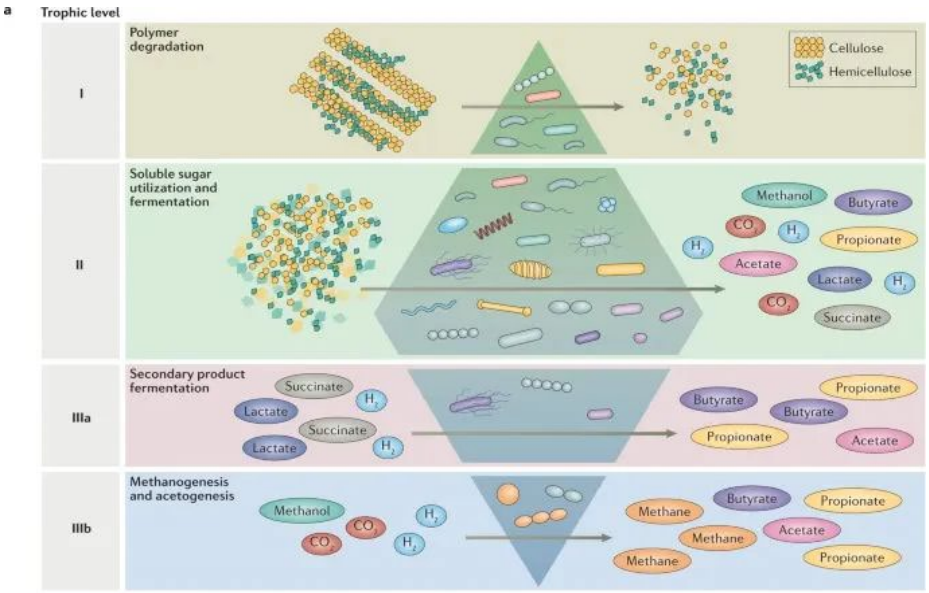

Figure 6. An overview of the rumen microbiota. Adapted from (Mizrahi *et al*., 2021).

The rumen is peculiar to ruminants and harbors complex microbiota, including bacteria, protozoa, archaea, and fungi, which together play fundamental roles in transforming plant material into consumable nutrients. Bacteria are the most diverse population of these ruminal microbes and have been the subject of extensive research in recent years (Fernando *et al*., 2010; Liu *et al*., 2016; McGovern *et al*., 2018). Age and diet affect the type of bacteria in the rumen (Jami *et al*., 2013). Several investigations have demonstrated that cattle fed a diet high in forage exhibited greater bacterial diversity than cattle fed a diet low in grain and that these prevalent diets contained different communities (Fernando *et al*., 2010; Plaizier *et al*., 2018; Liu *et al*., 2019).

Jami *et al*. (2013) mentioned a convergent bacterial community with advancing age and detailed the dynamic bacterial compositions at five representative stages from beginnings to maturity. Based on Yadav *et al*. (2016) reported that low ambient temperatures shortened the ruminal contents' mean retention period, which could lead to inadequate digestion. There are significant relationships between some genera and the apparent digestibility of fibre (Niu *et al*., 2017). There are still a few possible relationships between rumen bacteria, ambient temperature, and nutrient digestibility. Because it directly affects microbial growth, the ruminal pH is an essential ruminal characteristic.

According to Pereira *et al*. (2007), the pH range considered adaptive for microbiota is 5.5 to 7.0. It was possible to uncover the enormous diversity of digestive adaptations in other herbivores by applying methodological approaches and expertise from domestic ruminant research on "Ruminant-like" digestion in a Walla. (Moir *et al.,* 1954) offered possibilities for comparative research on foregut fermentation techniques. Consequently, the history of comparative digestive research has taken a two-fold top-down approach: (i) starting with what is arguably the most complex digestive system -ruminants-and moving on to fermentative digestion in numerous other vertebrates, such as fish and tadpoles; and (ii) starting with ruminants and moving on to the digestive physiology of numerous other ruminants, such as deer, antelope, and giraffe.

Foregut fermentation has been considered superior to hindgut fermentation due to observations of digestive efficiency in domestic herbivores. For this reason, the small intestine is the primary site of nutrient absorption, except for volatile fatty acids, which are primarily absorbed in the fermentation chambers (Alexander, 1993). In domestic (or "cattle-type") ruminants, the stratification of rumen contents is well-described (Hummel *et al*., 2009). Regarded as a “filter bed effect," a selective particle retention mechanism, it is to blame for the regional variations in papillating of the ruminal mucosa (Faichney, 2005). Considered an adaptation for contracting against a unique fibre mat, 'cattle-type' ruminants have forceful rumen pillars (Clauss *et al*., 2003). Conversely, ruminants that resemble mosses have rumen contents that are either less stratified or none, and their ruminal papillating even exceeds (Clauss *et al*., 2010). Animal eating habits, body composition, digestibility, rumen microbiota and methane emission, protein turnover, cellular and animal energy metabolism, endocrine system, and physical activity can all have an impact on the variation in feed efficiency of animals (Cantalapiedra-Hijar *et al*., 2018). These constitute only a handful of the numerous factors that form the biological factors associated with feed efficiency. Feed efficacy is impacted by rumen-related parameters, such as feed digestion and nutrient absorption. Compared to monogastric mammals, ruminants have developed unique morphological traits of a multi-chambered stomach, such as the rumen, reticulum, omasum, and abomasum (Chen *et al*., 2019).

The basic premises for digestion and fermentation of feed by the symbiotic microorganisms that create volatile fatty acids (VFA) are the primary end fermentation products in the rumen. The animals take nutrients, such as minerals and VFA, through the rumen epithelial wall and use them accordingly (**Figure 7**). The rumen epithelium absorbs more than 75% of ruminal VFA and uses it as an energy source (Na, 2022). To be more precise, there are multiple ways in which volatile fermentation products VFA are absorbed, including passive diffusion, nitrate-sensitive VFA absorption, proton-coupled dissociated VFA transport, bicarbonate-dependent transport, and electrogenic VFA transport (Penner, 2014).

The feed gets collected in the rumen, which has a pair of champers according to the specific weight and particle size; the ventral sac, in which sediments contain and digest roughage with a higher specified weight, and the dorsal sac, which collects gas and roughages with a lower specific weight because they are rougher and undigested (Öztürk and Gursel, 2021). The fermentation sac labelled as the rumen can hold up to 200 liters. Russell *et al*. (2009) calculated the entire volume of the rumen capacity of all domesticated ruminants worldwide, with a total volume of 100 billion liters. Rumen is the ‘‘largest commercial fermentation process in the world." Through the fermentation processes in the rumen, the microbes' enzymes significantly change the composition of the feed.

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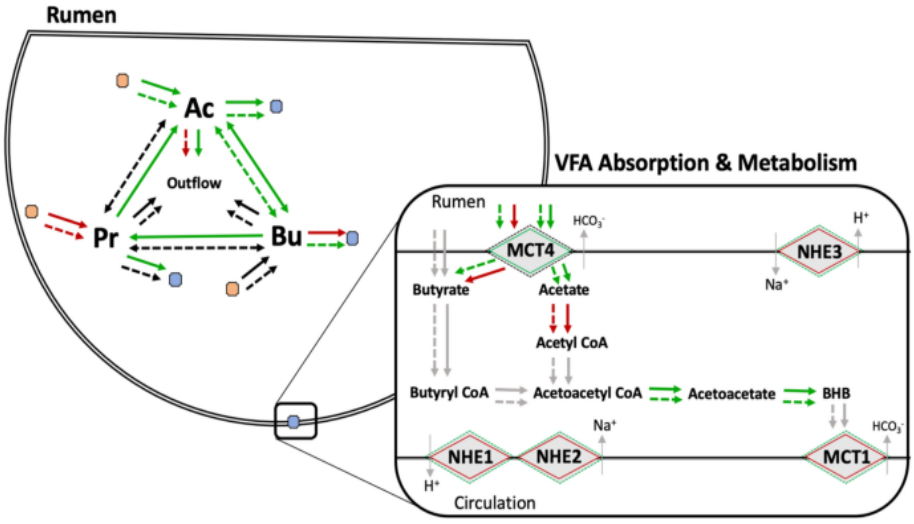

Figure 7. Ruminal volatile fatty acid (VFA) absorption. Adapted from (Bedford *et al.*, 2020)

The symbiotic bacteria in the rumen, not the ruminant were enzymes that carried out mechanical and enzymatic fermentation. Because of this phenomenon, ruminants have an advantage over other mammals and humans in that they can better digest or cannot digest nutrients like cellulose, hemicellulose, lignin, and pectin, which are the most prevalent (Naylor, 2012). The rumen microbial ecosystem demonstrated that, through systematic anaerobic culture methods, other inventions to bring food and diverse sectors to life depend on the microbial relationships with the host and each other (McSweeney and Mackie, 2012). The rumen acts as a place where the absorption of minerals, VFA, and nitrogenous substances takes place, along with the release of bicarbonate (Steele *et al*., 2016).

Indeed, the reticulum and the rumen fulfil the same purpose. Since it has an interior mucosa like a honeycomb, it has the vital task of moving the smaller, more densely digested particles into the omasum. The reticulum has a distinct anatomical form due to its two-stage motility, which is synchronized with the rumen and the omasum. The reticulum functions alongside the rumen to ensure that only the digested particles of the rumen content are transmitted to the omasum (Clauss *et al*., 2010).

The rumen has three main roles for effective fermentation: 1) maintaining ideal temperatures, pH levels, and osmotic pressure; 2) supplying the nutrients that microorganisms need to maintain the fermentation; and 3) ensuring that microorganisms adhere to particles through stirring. In fact, in this symbiotic interaction between ruminants and microbes, the microorganisms digest the forages because the rumen offers the best circumstances for growing the microbial population by eliminating VFA, which, if present in high enough amounts, reduces the pH of the rumen (Faverdin, 1999). Three groups constitute the rumen microbial community or microbiome, and there is considerable variability in the composition of the rumen (Cho *et al.,* 2006).

The fibre-adherent, planktonic, and epithelial (or epimural) related communities are among these groups. Subsequently, organic acids are the primary energy source for cow metabolism.

The rumen microbial community depends on the microbiome's natural adaptation and turns lignocellulosic substrates into short-chain fatty acids (SCFA) (Xiros *et al.*, 2019).

1.1 RUMEN EPITHELIUM AND CATTLE FEED EFFICIENCY

Feed efficiency is a crucial component of the prospective breeding goal for dairy cattle. Because of recognised variations in the morphological, physiological, and functional characteristics of the rumen epithelium, throughout its geographic range, the epimural microbiota at various ruminal locales varies (**Figure 8**). More specifically, the genera *Desulfobulbus, Butyrivibrio, Mogibacterium*, and *Campylobacter* dominate the epimural bacterial community (Mao *et al.,* 2015).

The profound stratification of rumen contents (McCowan *et al*., 1980) and the site-specific morphologies of ruminal epithelium tissue (Millen *et al*., 2016) predicted various bacterial populations depending on the location of the sample. These microorganisms break down resistant plant fibers and produce VFA, which supplies a significant amount of the host's energy needs (Flint *et al*., 2008; Purushe *et al*., 2010). Usually making up 15% of the animal's weight, the rumen's enormous volume extends the feed's retention period, enabling a more thorough feed conversion (Weimer, 2022).

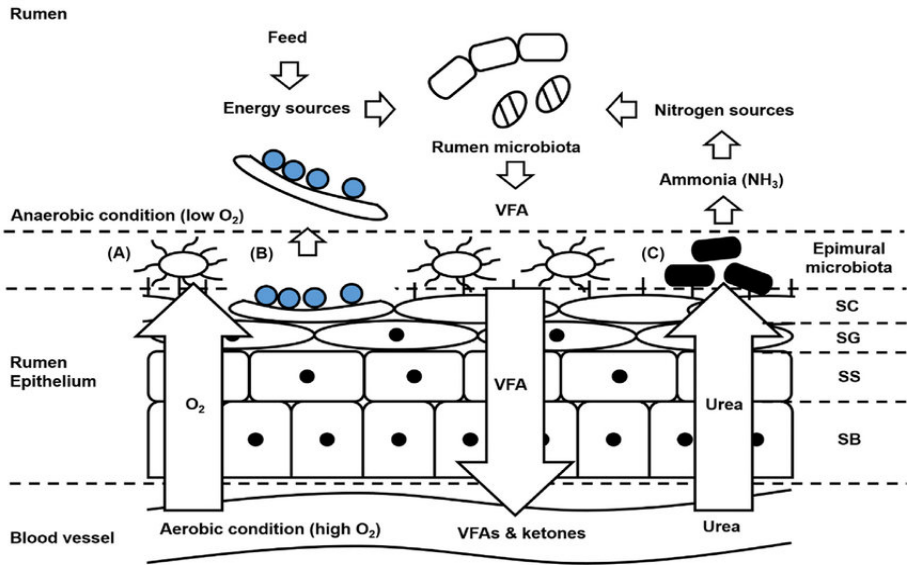

Figure 8. The rumen epithelium's shape and the role of the epimural microbiota: (A) urea digestion; (B) tissue recycling; and (C) oxygen scavenging. SS ¼ stratum spinosum; SB ¼ stratum basale; VFA ¼ volatile fatty acids; SC ¼ stratum corneum; SG ¼ stratum granulosum. Adapted from (Na and Guan, 2022).

In support of the hypothesis that the rumen has developed as an extension of the esophagus, rumen epithelium shares a gene expression pattern with esophageal epithelium rather than intestinal epithelium (Chen *et al*., 2019). The development of the rumen epithelium starts at the fetal stage (Na, 2022). Before birth, the rumen tissue of a 90-day-old bovine fetus differentiated

into mucosa, muscular, and serosa, with a stratified-cuboidal epithelium that included two distinct epithelial layers (basal and superficial; 10-cell layers). Neonatal rumen epithelium 30 days postpartum exhibited a more differentiated structure with six fewer cell layers, a stratified squamous epithelium (Nwaogu and Ezeasor, 2008). The amount of solid food and particles promotes greater keratinization, papillary growth, and rumen bulk.

The physical stimuli of firm feed promote rumen weight, and the chemical stimuli of fermentation products such as VFA promote papillary growth and keratinization and volatile fatty acids encourage papillary growth and keratinization (Baldwin and Connor, 2017). The metabolic activity of rumen epithelial cells varies in tandem with the morphological development of the rumen epithelium. (Lam *et al.,* 2018) demonstrated that the rumen epithelial tissues of efficient cattle were thicker, particularly the epithelium on the ventral blind sac, in contrast to inefficient cattle. Feed diet composition and fermentation products such as butyrate impact the thickening of the rumen epithelium associated with rumen thickness (Suarez *et al*., 2007; Diao *et al*., 2019).

Rumen developmental parameters like papillae length, papillae width, and rumen epithelium thickness would provide fundamental information as indicators and connectors between feed digestibility, rumen development, and feed efficiency, even though the relationship between rumen thickness and feed efficiency is still unclear. Furthermore, the rumen epithelium is involved in the passage of nutrients like urea and ammonia. Additionally, microorganisms in the rumen metabolize nitrogen sources found in food, producing ammonia and microbial proteins as byproducts. The rumen epithelium then absorbs ammonia in two different forms: lipophilic form NH_3 through passive diffusion or ionized form NH4þ via putative potassium channels, depending on the intraluminal pH (Abdoun *et al*., 2006). Moreover, the microbial breakdown of recycled urea from saliva or rumen epithelium can result in the formation of ammonia. As a byproduct of nitrogen metabolism, urea can be formed in the liver and then enter the rumen by saliva, passive diffusion through the rumen epithelium, and urea transporters on the rumen epithelium's basal and apical membranes (Getahun *et al*., 2019). An increase in the rumen epithelium's metabolic and functional activity may increase end products like b-hydroxybutyrate and acetoacetate, as well as have an impact on the expression of endocrine molecules in other tissues or organs that enhance systemic animal production, such as insulin (Manns and Boda, 1967), insulin-like growth factor-1 (IGF-1), and leptin (Kiani, 2013). Following weaning, rumen epithelial cells employ VFA-like butyrate as a primary energy source and ketogenic precursors, but in the milk-fed stage, all body tissues, including the rumen epithelium, use glucose as their primary energy source (Baldwin and Connor, 2017).

The rumen epithelium is essential for the absorption of VFA, the principal product of microbial fermentation, is absorbed via several methods, like bicarbonate-dependent transport, passive diffusion, nitrate-sensitive VFA absorption, proton-coupled dissociated VFA transport, and electrogenic VFA transport (Penner, 2014; Na and Gua, 2022). A stratified squamous epithelium that lines the luminal side of the rumen plays a role in defense, substantial short-chain fatty acid (SCFA) metabolism, transportation, and absorption.

According to Die *et al*. (2017), butyrate has been shown *in vitro* to influence nutrient absorption and other nutrient metabolism, as well as to start the process of tissue differentiation when solid feed is offered to a weaning newborn. More precisely, the *stratum basale*, *stratum spinosum*, *stratum granulosum*, and *stratum corneum* are the four cell layers that form the

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rumen epithelium (Baldwin and Connor 2017). The mouth, skin, and esophagus are examples of organs that frequently encounter the outside world and typically have a stratified squamous epithelium structure (Chruscik *et al*., 2021). The *stratum corneum* layer of the rumen epithelium acts as a protective barrier related to the oesophagus because of the widespread bacteria present in the rumen, and several papillae increase the surface area to effectively absorb nutrients, much like the intestine (**Figure 9**) (Meyer *et al*., 2014; Steele *et al*., 2016).

In the rumen, there are no microbial species with the total breakdown of the substrate. Therefore, a convoluted series of organisms participate in the joint breakdown of substrates and the generation of fermentation byproducts (Choudhury *et al*., 2015). The nutritional content affects the diversity, size, and activity of the microbial population in the rumen (Van Soest, 1994). However, animal-related factors like saliva production, rumen volume, and intake and passage rates (Pinares-Patiño *et al*., 2003; Hegarty, 2004), plus management factors like feeding essential oils have an impact (Patra and Yu, 2012). Three distinct parameters influence the amount of CH_4 emissions per unit intake: the rate at which organic matter degrades, the effectiveness of microbial growth, and the kind of VFA that is formed during the fermentation of organic matter (Czerkawski, 1986; Van Soest, 1994). The principal organism involved in the fermentation of carbohydrates in the rumen is bacteria (Hungate, 1966; Nagaraja, 2016). Cattle with high feed efficiency have more active tissue morphogenesis and VFA metabolism in their rumen epithelia, in addition to VFA absorption and nitrogen metabolism (Kong *et al*., 2016; Elolimy *et al*., 2018; Lam *et al*., 2018).

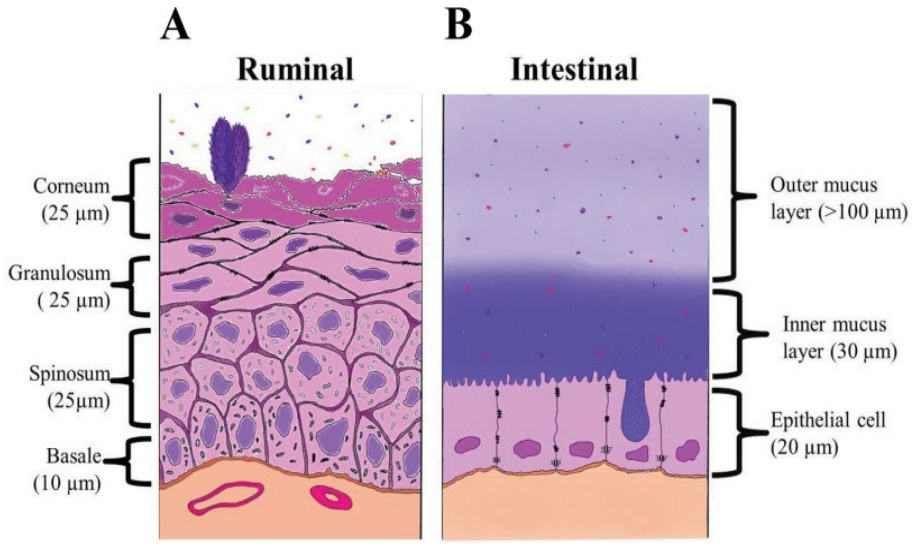

Figure 9. The fundamental distinction between the intestinal and ruminal epithelia. The rumen's stratified squamous epithelium (A) and the lower gut's columnar epithelium (B). Adapted from (Steele *et al.*, 2016).

Cattle with high feed efficiency have more active tissue morphogenesis and VFA metabolism in their rumen epithelia, in addition to VFA absorption and nitrogen metabolism (Kong *et al*., 2016; Elolimy *et al*., 2018; Lam *et al*., 2018). Prokaryotes (bacteria, archaea) and eukaryotes (protozoa, fungi) are among the remarkable diversity of microorganisms observed in the rumen, performing vital services for their host. A portion of these microbes have expertise

in breaking down plant polysaccharides, and as a result, they form an essential community that gives the host animal energy (White *et al*., 2014).

1.1.1 Rumen microorganisms and their role in ruminal fermentation

Each milliliter of rumen contains 10–50 billion bacteria, 1 million protozoa, and varying amounts of yeasts and fungi, making up the average rumen microbiota (Chellapandi *et al*., 2018). Rumen fermentation and the resulting H_2 supply and archaeal population are the two factors that lead to rumen methanogenesis (Belanche *et al*., 2015). Ruminal methanogens have a symbiotic connection with interspecies hydrogen transfer with other ruminal microorganisms, including ruminal protozoa, since they require the dihydrogen and carbon dioxide produced during ruminal fermentation (Dia *et al*., 2022).

A healthy ruminant requires a rumen microbiome of bacteria, fungi, and protozoa. In a paper by Langda *et al*. (2020), the rumen microbial community has evolved alongside the range of feeding techniques in different bovine lineages. Nutritional, adaptive, and physiological factors impact it. Connecting the microbiome composition, the functional roles of each species with cow phenotypes has proven to be a crucial problem in rumen microbiology (Bickhart and Weimer, 2018). The ruminant host cannot survive without the microorganisms due to their shared dependency, and certain rumen microorganisms (such as rumen protozoa) are exclusive to ruminants or very distinct from related microbes (such as methanogenic archaea) (Knapp *et al*., 2014). Methanogen abundance may not accurately indicate ruminant CH_4 emissions (Sejrsen *et al*., 2006). Since the balance between H_2 production (acetate and butyrate) and H_2 utilization (propionate) ought to be reflected in the molar proportions of rumen volatile fatty acids, rumen fermentation patterns ought to indicate variations in CH_4 emissions if rumen microbiomes are a significant contributing factor (**Figure 10**). According to estimates, the percentages of relative microbial domain biomass are as follows: 40% to 50% for protozoa; 26% to 42% for bacteria; 1% to 20% for fungi; and 0.3% to 4.0% for archaea (Wallace *et al.,* 2014; Tapio *et al*., 2017). The ruminant gut microbiome performs a list of distinct and physiological tasks considered necessary to preserve general equilibrium (**Figure 11**) (Singh *et al.,* 2019; Stockler *et al*., 2022).

Ruminant production efficiency depends on the bacterial population in the rumen microbiota. These bacteria facilitate the creation of microbial protein and convert indigestible plant biomass into energy (Firkins, 2010). Upgrades in feed efficiency and milk production could result from a better understanding of the relationships between microbiological regulations, especially in dairy cows (Weimer, 2015). Despite their small proportion, bacteriophages and parasites may nevertheless impact the microbial diversity and, in turn, the efficiency of rumen fermentation (Fox *et al*., 2018). While several factors affect the rumen's microbial diversity, the feed's composition is the most critical component since it modifies the rumen environment through impacts on microbial diversity (Krehbiel, 2014).

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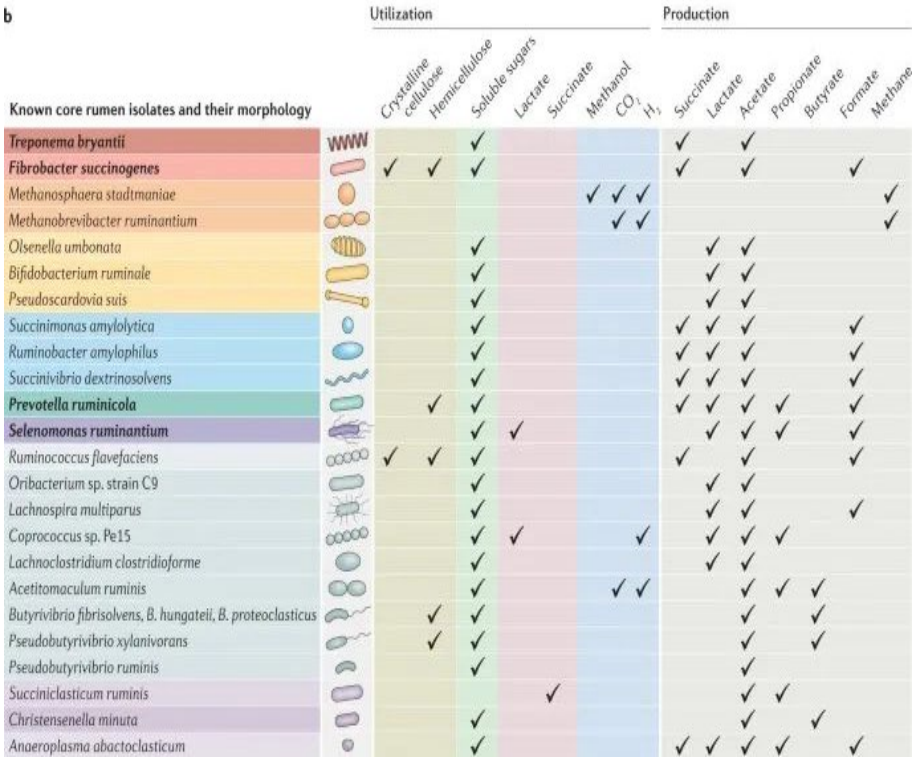

Figure 10. Principal microorganisms in the rumen and the end products generated there. Adapted from (Mizrahi *et al.*, 2021).

Indeed, there is an outside cause that could alter the rumen; all biochemical flux relies on the structure of the feed, the variety of rumen microbes, and the ambient parameters of the rumen. As a result, even if it is simple to modify the feed's composition and add additives to improve the rumen's fermentation and microbial diversity, the rumen microorganisms take some time to adjust to the new environment after receiving different feed and additives (McAllister and Newbold, 2008). *Actinobacteria* are gram-positive bacteria that can break down starch and polysaccharides that resemble starch as oligosaccharides. The main metabolic byproducts of this process are lactate and acetate (Ventura *et al*., 2007).

Plant material must be settled by a complex microbial consortium in the rumen for it to degrade. This process happens in a period that depends on and varies by the type of substrate consumed (Elliott *et al*., 2018). Though variables altering the rumen epithelium's gene expression and potentially influencing the rumen outflow or volume have been proposed, the exact methods by which the host might regulate the rumen microbial population are yet unknown (Huws *et al*., 2018).

Although protozoa are not believed to be actively involved in biohydrogenation, bacteria are primarily responsible for the process in the rumen (Lourenço *et al*., 2010). Yet, protozoa do have an impact on the constitution of the rumen bacterial population and may thus be involved in biohydrogenation (Newbold *et al*., 2015).

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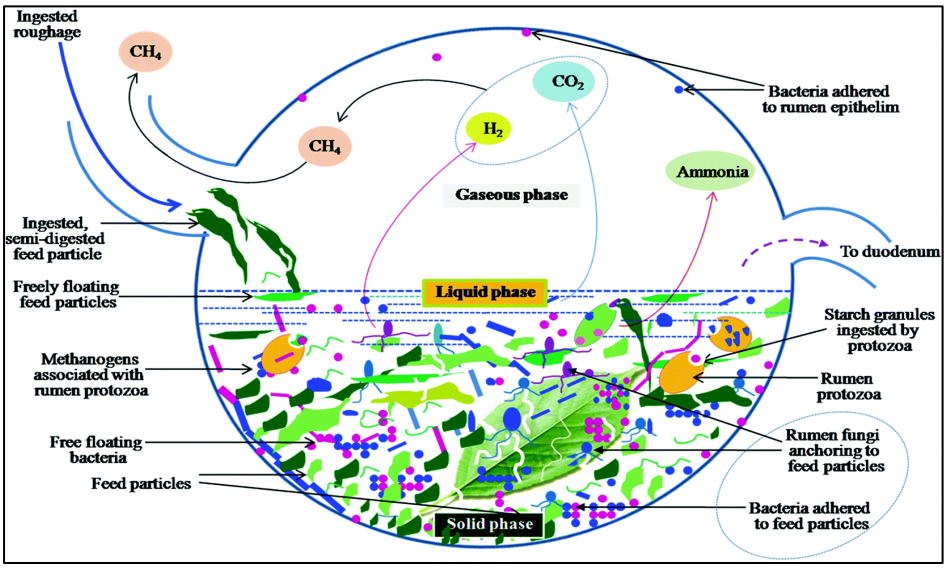

Figure 11. Diagrammatic representation of the rumen and its constituent phases the solid, liquid, and gaseous phases. Methanogens use the surplus H_2 generated in rumen-by-rumen fungus and protozoa to make methane. Methane escapes the mouth and enters the environment. In the abomasum, the microbial biomass generated in the rumen is broken down. Adapted from (Singh *et al.*, 2019).

1.1.2. Microorganisms of epimural microbiota

Since the 1960s, researchers have examined the composition and function of the rumen epithelial attached microorganisms, or epimural microbiota The rumen epithelium facilitates this colonization (Rahman and Decker, 1966; Wallace, 1979). These microorganisms can be divided into different groups:

1.1.2.1 Rumen bacteria

Gregorini *et al.* (2015) state that the rumen modelling has focused on how feeding affects emissions and efficiency rather than the bacteria and their roles. A recent study by Islam *et al.* (2019) showed the rumen microbiome performance of bacteria and viruses related to three microbial groups: *Firmicutes*, *Bacteroidetes*, and *Archaea*. Researchers in microbiology adopted culture-based techniques to identify distinct members of the rumen bacterial community; these techniques are based on early research (Hungate *et al.*, 1971; Krause *et al.*, 2013). The rumen microbiota and its fermentation products are linked to feed efficiency and CH_4 emissions (O'Hara *et al.*, 2020).

Additionally, they observed that the predominant bacterial phyla that can ferment a broad range of dietary sugars and peptides are *Firmicutes, Bacteroidetes,* and *Proteobacteria*. The global biogeochemical cycles induced by microorganisms are arguably widespread and ancient forms of life on Earth. They may be found in almost every ecosystem (Louca *et al.*, 2019). In cattle, strict anaerobes predominate in the ruminal bacterial community. *Eubacterium, Bacteroides, Clostridium,* and *Prevotella* were the most common ruminal genera. They release enzymes that aid plant polymers in fermentation and breakdown (Xu *et al.*, 2021).

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The breakdown of the ingested fibre is made possible by the collective interaction of several bacteria. It is now simpler to research the function of commensal flora in host metabolism using advancements in next-generation sequencing technologies, microbiological methods, culture-free methodologies, and genetic engineering. Plant cell walls are constructed of a hemicellulose matrix with cellulose fibers inserted in it (**Figure 12**) (Ungerfeld and Emilio, 2020).

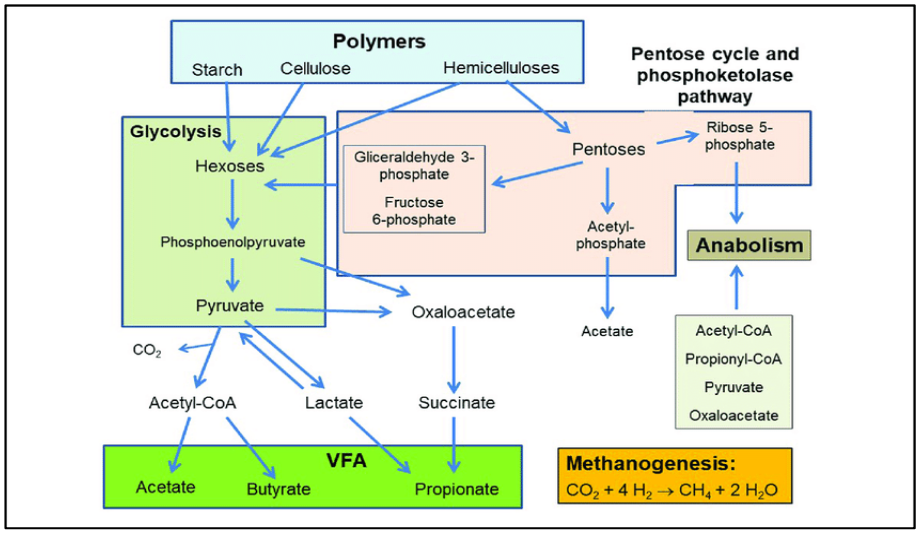

Figure 12. Simplified scheme of carbohydrate fermentation in the rumen. Adapted from (Ungerfeld and Emilo, 2020).

A specific taxon of bacteria secretes cellulolytic enzymes, which are responsible for the first destruction of this matrix (Flint and Bayer, 2008; Flint *et al*., 2008). Most xylanase and endoglucanase activity in the rumen is related to bacteria. These break down cellulose into smaller oligo/disaccharides, processed by other living things. The Gram-positive phylum comprises first-order cellulolytic called *Ruminococci*. Firmicutes include *Ruminococcus albus, Ruminococcus flavefaciens*, and *Fibrobacter succinogene* (Russell, 2002). In addition, compared to the mentioned organisms, *Butyrivibrio fibrisolvens* are found at a limited level. Cellulolytic bacterial end products serve as substrates for various microflora, initiating additional breakdown of these substances (Weimer, 2022). Whereas *Lachnospira multiparus* and *Succinovibro dextrinosolvens* break down pectin, *Selenomonas ruminantium*, *Succinomonas amylolytic*a, *Butyrivibrio fibrisolvens*, *Streptococcus bovis, Ruminobacter amylophilus, and Prevotella* species hydrolyze other essential polymers like starch. Moreover, the type of diet consumed affects the composition of the bacteria (Erickson *et al*., 2002; Krause *et al*., 2003; Mitsumori *et al*., 2003).

It has a significant effect on the composition of the microflora in the gut environment and evaluates the kind of bacteria needed to digest the food consumed (Callaway *et al*., 2010). Most of the bacteria in the rumen are fermenters of sugar and starch. The polysaccharides found in plants are optimally harvested in terms of energy since many other species utilize the end products of bacterial fermentation as a substrate. Emerging metagenomic investigations on the gut microbiota of many mammalian species have demonstrated that. In comparison to

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carnivores, the microflora of ruminants and herbivores contain a greater abundance of the anabolic pathways that produce amino acids.

The amino acid (AA) production pathways in the rumen microbiota, in addition to having proteolytic activity, several species, like *B. fibrisolvens, P. ruminicola, S. ruminantium, and R. amylophilus*, also have a certain amount of cellulolytic activity. *P. ruminicola* produces more ammonia (NH_3), deaminase, and proteolytic activity in the rumen. Since less protein and ammonia are present in the rumen environment, which serves as nitrogen sources for AA and protein synthesis, this activity is essential (Stevenson and Weimer, 2007). Sulfate-reducing bacteria are among the several groups of bacteria that help convert sulfate to H_2S. The rumen microbiota is adjusted based on dietary modifications to promote the breakdown and fermentation of different complex chemicals.

Moreover, they interact communally with the host and one another to guarantee both their survival and optimal energy generation. They also contribute to the host organism's supply of proteins and VFA. Variations in microbiota concentrations can occasionally affect the host system and may result in illnesses (Xu *et al*., 2021). *Enterococcus* and *streptococcus* species colonize the first floor of the neonate's rumen, converting the gut environment to anaerobic conditions (Jami *et al*., 2013). The level of rumen bacteria activity directly correlates with the dairy cow's capacity for digestion. While the culturable biota has undergone extensive study, several of the species found in the rumen are non-culturable (Malmuthuge *et al*., 2013). Research shows that *Prevotella* (15.1%) and *Bacteroidetes* (15.8%) make up the microflora of 21-day-old calves, suggesting that the formation of the rumen microbiome is driven by starting feed during maturation.

The population of 3-5 day 5-day-old methanogens and cellulolytic members is comparable to that of adult mammals (Xu *et al*., 2021). Remarkably, compared to adult organisms, the rumen microflora of 14-day-old calves contains a greater diversity of transient bacteria. Then, rumen bacteria can divided into four essential subpopulations: 1) those attached to the rumen epithelial cells, which make up less than 1% of all rumen microbes; 2) those attached to feed particles, which make up approximately 70-80% of all rumen bacteria; 3) those suspended in the ruminal fluid, which makes up approximately 20–25% of all rumen bacteria; and 4) those attached to the surface of protozoa or fungal sporangia (Zhou *et al*., 2015). The rumen was the site of around 3,000 different types of bacteria (Attwood *et al*., 2008a). Obligate anaerobes constitute a substantial portion of the rumen bacterial population (Bansal and Goel, 2015).

Consequently, it is fundamental to maintain the rumen O_2 content below 0.5% to survive the rumen anaerobic bacteria. A negligible proportion of the bacterial population called facultative anaerobes plays a critical role in keeping the rumen environment at anaerobe conditions by absorbing oxygen from feed input. For those germs to stay alive, they need a tiny bit of oxygen. Most bacteria in the rumen are gram-negative. Conversely, the count of Gram-positive bacteria rapidly increases in ruminant-fed concentrates (Hernández *et al*., 2014).

As a result, the organization of rumen microbes alters due to variations in diet composition. Transient alterations in the diet can also negatively impact bacterial and protozoan species, potentially causing pathogenic issues. The increased concentration of concentrates in the feed is particularly beneficial for producing lactic acid, hyper-ammonia-producing bacteria that can produce ammonia 20 times faster than other species, methanogenic archaea, Gram-positive rumen bacteria, and hyper-ammonia-producing bacteria (Chen and Russell, 1989). While Gram-negative bacteria create propionate and succinate, which are far more suited for

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ruminants, Gram-positive bacteria also produce hydrogen, butyrate, acetate, lactate, format, and other compounds (Underwood *et al.,* 2015).

Fiber-associated bacteria play a crucial role in the fermentation and breakdown of plant components. Before ingesting solid feed, the undeveloped rumen of lambs contains bacteria and archaea, with levels comparable to those observed in adult animals approximately ten days postpartum (Yáñez-Ruiz *et al*., 2015). Rumen bacteria can be categorized by function into:

- **Cellulolytic**.
  Fibrobacter succinogenes, Butyrivibrio fibrisolvens, Ruminococcus flavefaciens, Ruminococcus albus, Clostridium cellobioparum, Clostridium longisporum, Clostridium lochheadii, and Eubacterium cellulosolvens are among the essential bacteria observed in the rumen environment. Three cellulolytic bacteria of the rumen, Bacteroides *Fibrobacter succinogenes*, *Ruminococcus albus,* and *Ruminococcus flavefaciens,* are the most significant (Hungate, 1950; Choudhury *et al*., 2015).
- **Hemicellulolytic**.
  The rumen contains non-cellulose fiber-degrading bacteria called *Prevotella ruminicola, Eubacterium xylanophilum*, and *Eubacterium uniformis*. More than a hundred glycoside hydrolases are present in them (White *et al*., 2014; Choudhury *et al*., 2015).
- **Pectinolytic**.
  Of all the bacterial species in the rumen, the three previous groups comprise an enormous group. The primary degraders of pectin are *Treponema saccharophilum*, *Lachnospira multiparus, Bacteriodes ruminicola, Succinivibrio dextrinosolvens, Butyrivibrio fibrisolvens, and Streptecoccus bovis* (Choudhury *et al.,* 2015).
- **Amylolytic.**
  The primary amylolytic bacteria are *Butyrivibrio fibrisolvens (*Cotta, 1988), *Prevotella ruminicola* (Choudhury *et al*., 2015), and *Ruminobacter amylophilus* (Demirtaş, 2013). *and Streptococcus bovis* (Chesson and Forsberg, 1997). *Saccarolytic microorganisms include Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus fermentum, Bifidobacterium ruminantium, Selenomonas ruminantium, and Succinivibrio dextrinosolvens*.
  Rumen bacteria, on the other hand, do not adhere strictly to a single biochemical flux. Instead, a variety of roles are combined, as seen by the bacteria listed above. For example*, Prevotella ruminicola* and *Butyrivibrio fibrisolvens* are hemicellulolytic and pectinolytic, respectively. *S. bovis* is the most robust to pH 4.5, while amylolytic bacteria in the rumen are more resilient to lower pH levels (Therion *et al.,* 1982; Öztürk and Gursel, 2021). When ruminants are fed a substantially higher grain diet, the growth of amylolytics is encouraged. Compared to cellulolytics, amylolytics proliferate, doubling between 15 minutes and 4 hours. As byproducts, these species generate format, succinic acid, lactic acid, propionic acid, and CO2. They have to do with metabolizing soluble sugar and starch (Öztürk and Gursel, 2021).
- **Acetogens**.
  It is a tiny subset of rumen bacteria that can produce reductive acetogenesis from CO_2 and H_2. There should be more acetogens in the rumen, specifically *Acetitomaculum ruminis* and *Eubacterium limosum* (Joblin, 1999).

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- **Proteolytic**.
  The most prevalent proteolytic organisms are *Clostridium bifermentans, Colostridium proteoclasticum, Ruminobacter amylophilus, and Prevotella ruminicola* (Choudhury *et al*., 2015).
- **Users of acid and urea**.
  Megasphaera elsdeni and *Anaerovibrio lipolytica* play a vital role in situations where the high grain content of the diet causes a high generation of lactic acid in the rumen. Ammonia absorbed across the ruminal wall accounts for up to 50% of the total ammonia flow to the liver, and the primary cause of low efficiency of N utilization is the urine excretion of urea synthesized from ammonia lost from the rumen (Tamminga, 1992; Vaga, 2017).
- **Lipolytic.**
  Anaerovibrio lipolytica may use lactic acid in ruminants on a high-grain diet. Every fermentation process releases some H_2 (Stewart *et al*., 1997).

1.1.2.2 Rumen methanogens (*Archaea*)

Rumen Archaea are microorganisms that combine carbon dioxide and hydrogen from metabolism to make water and methane (**Figure 13**).

Therefore, by generating methane, the archaeal helps protect the rumen from too much hydrogen (Garnsworthy *et al*., 2019). The amount of CH_4 formed is dependent on the diversity and abundance of methanogenic archaea as well as their interactions with other microbiota, even though they make up less than 5% of the total ruminal microbiota (Janssen and Kirs, 2008; Pitta *et al*., 2016b). CH_4 production causes a significant energy loss for the ruminant. By converting H_2 and CO_2 into CH_4, methanogenic archaea had a profound impact on pH regulation and H_2 partial pressure modulation in the rumen. There is a connection between the rate of digestion of the fibre and the formation of CH_4 due to this interspecific hydrogen transfer (Løvendahl *et al.,* 2018). The investigation into archaea relationships with CH_4 generation was prompted by the organisms' recognized methanogenic potential (Wallace *et al.,* 2014).

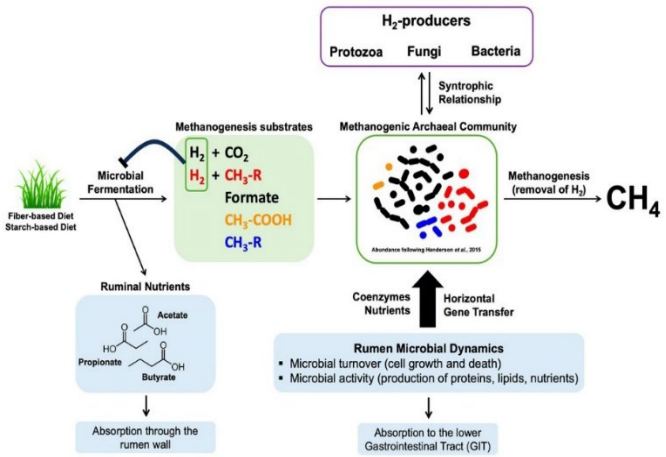

Figure 13. The metabolic adaptability and ecophysiology of rumen methanogens. An outline showing the functions of methanogens, which remove H_2 from microbial fermentation to produce methane and allow rumen microbial fermentation to continue. Adapted from (Khairunisa *et al*., 2023).

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Considering *Archaea* almost exclusively relies on methanogenesis for ATP synthesis, it is argued that Archaea biomass yield should be proportionate to CH_4 generation (Wallace *et al.,* 2014). With an estimated 0.6–3.3% of the entire rumen microbiota, anaerobic methanogens comprise most archaea (Yanagita *et al.,* 2000). Euryarchaeota is the grouping under Archaea. They are *methanobacteriales, methanopyrales, methanococcales,* and *methanosarcinales.* The bulk of ruminant methanogens are classified into one of the three groups.

Within each rumen, the predominant archaea species was the same. Nearly all species in the rumen are determined to be *Methanobrevibacter* species. Methanogenic communities demonstrated resilience to food variations, and compared to primiparous cows, methanogenic groups in multiparous cows were marginally more diversified. Methanogenic communities remained unchanged when the concentration proportion in the diet was increased from 25 to 50% (Mao *et al.,* 2016). While Jami and Mizrahi, (2012) and Li *et al.* (2012) have reported variations in the abundance and variety of bacterial communities' comparable information is absent regarding methanogenic communities.

Recent research has shown that rumen methanogens belong to the domain's archaea and make up 2.8–4% of ruminal microorganisms (Janssen and Kirs, 2008; Su *et al*., 2014; Morgavi *et al.,* 2010). It is generally agreed upon that the rumen microbiome comprises just a fractional percentage (3%) of Archaea communities (Belanche *et al.,* 2014). *Methanobrevibacter* makes up more than 60% of the rumen archaea; *Methanomicrobium* can make to 15%; and the remaining rumen Archaea are called rumen cluster C.

About 16% of the rumen Archaea are *Thermoplasmatales,* whose role in the rumen is unknown. As the primary CH_4 producers in the rumen, rumen methanogen archaea have an impact on rumen fermentation despite being a tiny number of rumen microorganisms. Research on the connection between ruminal protozoa and *Archaea* is growing, especially spotlighting the need to create enteric methane mitigation plans (Boadi *et al.,* 2004). The symbiotic link between methanogenic archaea and protozoa has been well-established (McAllister and Newbold, 2008; Morgavi *et al.,* 2008; Mosoni *et al.,* 2011).

It also accelerates the pace of protozoal substrate fermentation by generating methane from H_2 extracted from reducing equivalents. According to some research, protozoa may preferentially consume starch granules over fibre particles due to the presence of methanogens. Additionally, due to the elevated degree of bacterial competition for starch, amylolytic bacterial populations seem to evolve more rapidly in animals (Kurihara *et al.,* 1978).

1.1.2.3 Rumen protozoa

Certain ciliate protozoa inhabit the rumen environment of bovines and provide 40% to 50% of the rumen microbial biomass, nearly matching the total quantity of rumen bacteria (**Figure 14**) (Sylvester *et al.,* 2004; Gruninger *et al*., 2019).

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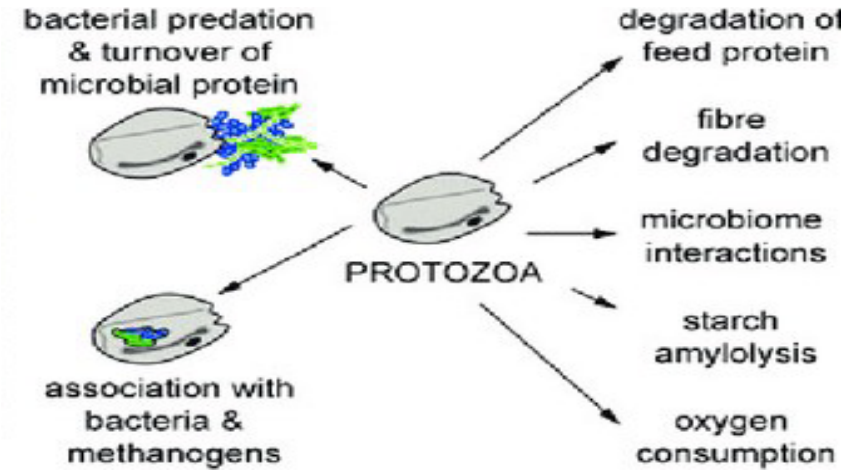

Figure 14. The community of the rumen microbiome, protozoa carry out a few crucial tasks. Adapted from (Gruninger et al., 2019)

Traditionally, taxonomy and protozoal identification have depended on optical microscopy-based morphologic identification (Newbold *et al.,* 2015). On the other hand, ciliates survive by digesting rumen bacteria, which is a significant factor in ruminants' inefficient use of dietary protein (Morgavi *et al.,* 2010). Because rumen ciliates and prokaryotic cells have a symbiotic relationship and are predator-prey, rumen protozoa are vital for feed digestion and maintaining the equilibrium of the rumen environment (Newbold *et al.,* 2015).

As evidenced by the defaunation or eradication of certain protozoa, the robust functional relationship between rumen protozoa and methanogens through interspecies hydrogen transfer contributes to the rumen's total CH_4 production (9%–37%) (Hook *et al.,* 2010). Protozoa in the rumen have evolved unique roles tailored to their survival (Dehority, 1991). Though very few species can sequester oxygen, nearly all protozoa are anaerobic. It keeps the reticulorumen's anaerobic atmosphere intact, and oxygen sequestration from the surroundings benefits the host. Furthermore, it aids in the digestion process and helps strict anaerobes survive. Protozoa use complex carbohydrates, like lignocellulose, starch, and sugar, to produce energy. Over half of the biomass found in the rumen is made up of ruminal protozoa (Belanche *et al.,* 2011). Instant engulfing facilitates the degradation of lipids, proteins, and carbohydrates. (Stewart *et al.,* 1992).

The lateral gene transfer from the bacteria that protozoa ingest results in their ability to digest lignocellulosic materials (Ricard *et al.,* 2006). Ciliates are essential to fermentation and the breakdown of plant fibers. The fermentation products of protozoa bear similarities to those of bacteria. Protozoa divide at a slower rate (15–24 h) than bacteria. They often live at lower rumen levels to withstand the washing out of protozoa before division. Protozoa can absorb and hold onto great amounts of starch simultaneously, which lowers pH and prevents acid production (Ranilla *et al.,* 2007). Plant sugars and fibers are degraded by proteases (Holotrich), which also produce pectin esterase, invertase, amylase, and polygalacturonase.

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In smaller amounts than entodiniomorphids, protozoa also generate cellulolytic and hemicellulolytic bacteria. Proteases secreted by ciliates in the rumen cause the synthesis of ammonia and amino acids. There is growing evidence about the connection between methanogenic archaea and protozoa, as well as their significance in the growth of rumen CH_4 (Malmuthuge and Guan, 2017; Wallace *et al.,* 2017). Ruminal protozoa engage in methanogenesis by producing acetate and butyrate, which release two and four pairs of H atoms per mole of fermented glucose, respectively. *Entodiniomorphids* and *isotrichids* are the two groups of ruminal protozoa. Because they consume more oxygen, *isotrichids* have distinct endosymbiotic methanogens compared to *entodiniomorphids* (Belanche *et al.,* 2014), as well as have a more potent impact on ruminal methanogenesis (Belanche *et al.,* 2015). The capacity of ruminal protozoa to create H_2 in their hydrogenases and host and endosymbiotic methanogens while shielding them from oxygen toxicity may play a role in methanogenesis (Fenchel and Finlay, 2006).

The role of isotrichids in methanogenesis exceeds that of entodiniomorphids (Dai *et al.,* 2022). In the rumen, protozoa are unicellular creatures held in place by a pellicle or cuticle. They represent the most basic eukaryotic organisms in the universe. Because they consume organic materials, bacteria, and cell waste, most species of protozoa are parasitic. Compared to various flagellates, ciliates are more common in the gut of ruminants. Around 25 genera make up the *Vestibuliferida* and *Entodiniomorphida* subcategories of ciliates. The type of ingested microbiota determines the protozoa's nitrogen metabolism. The production of nitrogenous compounds impacts nitrogen recycling. Rumen ciliates have an impact on both ammonia production and VFA synthesis.

It is indisputable that protozoa have a variety of host metabolic systems (Sirohi *et al.,* 2013). Apart from bacteria, another type of microorganisms found in the rumen are the facultative or obligate anaerobic protozoa, which include different types of flagellates and ciliates. Nearly half of the biomass in the rumen is made up of cilia, while flagellates make up far less. (Öztürk and Gursel, 2021) propose that protozoa may play a role in the digestive processes of the rumen. However, according to some experts, they are only a supply of nitrogen to make up for the low-quality feed that wild ruminants receive during the off-season (Yokoyama and Johnson, 1988).

They are essential in avoiding rumen acidosis because they quickly eliminate readily fermented carbohydrates. Additionally, they lessen the chance of heavy metal toxicity and remove harmful substances from plants. Protozoa are unnecessary for the rumen's typical digestion processes (Nagaraja, 2016). The protozoa population has an essential effect on the rumen's CH_4 production because of the interspecies transfer of hydrogen from the protozoa to methanogens (Li *et al.,* 2017). Additionally, the amount and activity of the methanogen endosymbiotic population outweighed that of the ectosymbionts affixed to the surface of protozoa (Morgavi *et al.,* 2010). The composition of the methanogen community, rather than variations in methanogen cell density, is primarily responsible for the variation in CH_4 emissions that arise from the microbial modifiers or plant secondary chemicals (St-Pierre *et al.,* 2015). Certain protozoa species might influence rumen methanogenesis and CH_4 synthesis more than others (Tan *et al.,* 2020). Furthermore, protozoa incorporate unsaturated fatty acids directly, preventing biohydrogenation in the rumen and facilitating direct transfer into milk and meat (Lourenço *et al.,* 2010). Up to 33% more methane output (g CH_4/kg of DMI) is produced when intestinal parasite infestations are present (Fox *et al.,* 2018).

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1.1.2.4 Rumen fungi

Twenty per cent of the rumen microbiota is formed of fungi. The contribution of anaerobic fungi to the rumen's microbial biomass is a topic of significant controversy. According to Newbold and Ramos-Morales (2020), the vegetative growth of rhizoids on and in plant material is less evident than the flagellated zoospores, which are apparent in rumen fluid. Anaerobic fungi can degrade the lignocellulose found in rumen (**Image 1**).

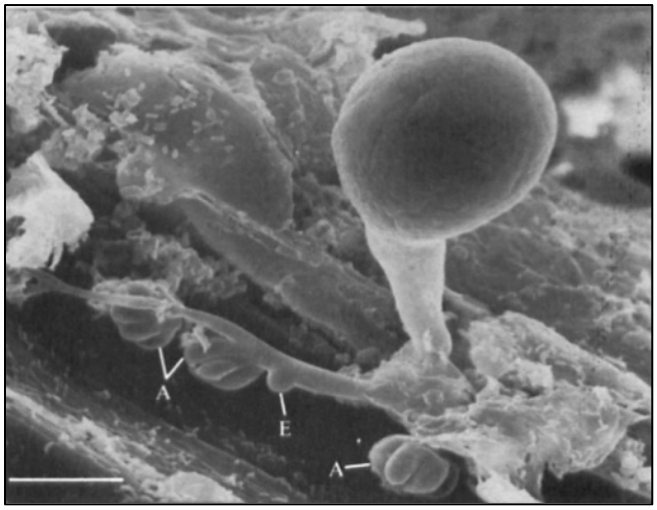

Image 1. The rumen fungus in the cow rumen displays a rhizoid structure with developing (A) and emerging (E) "appressoria" in the cell lumen of the grass. Adapted from (Ho *et al.*,1988).

Protease-producing fungi also display fungal structure, a polycentric or monocentric thallus, and polyflagellate or uniflagellate zoospores. A great deal of anaerobic fungi's asexual life cycle is evident (Dehority and Tirabasso, 2000). Though there is an ongoing discussion on the taxonomy of rumen fungi, six genera are generally accepted: the polycentric *Anaeromyces, Orpinomyces*, and *Cyllamyces,* and the monocentric *Neocallimastix, Caecomyces*, and *Piromyces* (Edwards *et al.,* 2017). The fungi are vividly visible throughout the animal's digestive tract and reside in the rumen. Additionally, fungi were isolated from the dairy cow's saliva and excrement. The rumen can retain more food due to the presence of these organisms, around 8% of the total ruminal microbiota in animals fed forage (Rychlik and Russell, 2002). However, the fungal population declines with high-grain diets. Fungal cultures release enzymes that break down cellulose, hemicellulose, lignin, and starch (Jami *et al.,* 2013).

Fungi are also stringent anaerobes, meaning that the only way to produce energy is through the fermentation of carbohydrates. The co-players of oxidative phosphorylation, mitochondria, and cytochromes are absent from fungi. They nevertheless have hydrogenosomes, which help in energy production. Fungal genera are not the only organisms that have evolved into hydrogenosomes, which are derivatives of mitochondria. With *Megasphera elsdenii, Selenomonas ruminantium*, and *Viellonella alcalescens*, the fungi's power to degrade cellulose increases significantly. Compared to the bacterial activity, the fungal breakdown of cellulosic materials seems less active. According to Bernalier *et al.* (1993), rhizoids penetrate plant cell walls (**Image 2**) and facilitate easy access to lignocellulosic material for the rest of the rumen microbiota. As a result, they demonstrate extraordinary activity in this regard. All fungi found thus far are required to be anaerobic, and their ideal temperature range is only 33- 41 °C (Öztürk and Gursel, 2021).

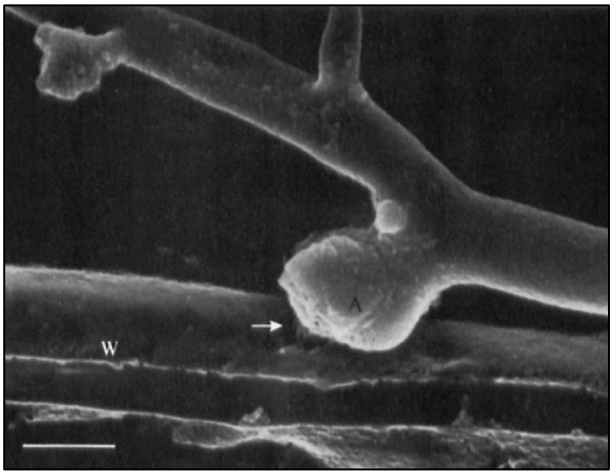

Image 2. The penetration peg and the cell wall come into contact, take note of the little hole or channel (arrow) created there. Appressorium (A) from the cattle rumen, with a penetration peg piercing the rice straw's cell wall (W). Adapted from (Ho *et al.*, 1988).

The rumen is home to species of fungi that are members of the genera *Neocallimastix, Piromyces, Orpinomyces, Caecomyces,* and *Anaeromyces*. Fungi exhibit wide variations in nutrition, peaking in the ruminal population 12 hours post-meal. When fed roughage, their mass rises to 8% of the overall biomass (Thirumalesh and Krishnamoorthy, 2013). Due to their ability to break down plant cell walls and promote digestion, fungi also aid in the growth of bacteria on plant structures. According to reports, a few fungal species have demonstrated proteolytic activity. Reduced fungal growth is another benefit of high quantities of easily digested carbohydrates. This explains why fungi in roughage-rich feeding are becoming more numerous (Orpin and Joblin, 1997).

2.1.2.5. Rumen viruses

The presence of viruses in the rumen of domestic animals was first editorialized in the 1960s (Adams *et al.,* 1966; Hoogenraad *et al.,*1967; Edwards *et al.,* 2021) and it was quickly recognized that these viruses were not just those ingested by chance from feed and water (Gilbert *et al.,* 2020). Instead, most viruses in the rumen are those that either co-exist or actively replicate and predate on the dense microbial populations of the rumen (Anderson *et al.,* 2017).

Although rumen-associated viral populations have received far less attention in research to date than other microbial populations, they may play a main role in maintaining the balance of microbial populations through intra-ruminal microbial lysis, fiber breakdown, nutrient cycling, and genetic transfer (Gilbert *et al.,* 2020). The review by Cilbert *et al.* (2020) offers a fresh viewpoint and enhances our comprehension of the functions that viral populations play in the rumen microbiome by summarizing our current knowledge of rumen virus populations. Rumen microbiome functional stability facilitates genetic exchange and allows nutrient cycling (**Table 1)** (Gilbert *et al.,* 2020). Viruses called bacteriophages are responsible for infecting rumen bacteria. Per milliliter of rumen fluid, their numbers vary from 2^{x107} to 1^{x108}. Even though there are currently over 100 distinct bacteriophages identified, considering that bacteriophages are exclusive to all bacterial species, it is reasonable to assume that this number will increase to include a significant number of bacteriophage species.

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It is possible to deduce the effects of viral activity in the rumen based on the biology of individual viral isolates and several significant characteristics of rumen virus populations that have been created. It is a well-established fact that most rumen viruses are tail viruses, which belong to the class of viruses known as *Caudovirales*. These prokaryote viruses, also known as bacteriophages, are the most researched and well-understood because they are consistently present in large quantities and actively replicate in the dense bacterial communities of the rumen. Any changes in the animal diet and the resulting availability of nutrients for microbial growth in the rumen are expected to cause significant changes in the relative abundance of viral groups since viral populations are connected to the populations of their microbial hosts (Gilbert and Klieve, 2015).

1.2. FACTORS AFFECTING METHANE PRODUCTION

According to Rojas-Downing *et al.* (2017), there is a projected increase in demand for animal products in the future. Consequently, if production efficiency remains unchanged, there will be a significant rise in GHG emissions. There will be a rise in demand for items due to the expected improvement in living standards for lower- and middle-class populations (Enahoro *et al.,* 2019). However, animal weights and the corresponding amounts and components of animal products may vary due to feed fluctuations and seasonal variations in the climate (Nantapo and Muchenje, 2013). As a result, seasonal variations in emission factors and the final CH_4 emissions from enteric fermentation may result from these changing conditions. For cattle production systems without supplemental feeding, seasonal variations in pasture quality and fodder availability lead to variations in dry matter intake, which leads to irregular emission factors (Van Wyngaard *et al.,* 2018). Numerous factors, including grass type, chemical composition, concentrate ratio in the feed, the rate at which digested food passes through the rumen, physiological stage, breed, and sex, affect CH_4 yield. It assumed that factors are all integrated into the single expression of DMI (Archimède *et al.,* 2018). The external environment can enter the rumen, which is a dynamic system. However, the ruminal microbial niche remains stable and provides an ideal setting for the growth and multiplication of the diverse anaerobic rumen microbial community. The main variables influencing these bacteria' development and activity include temperature, pH, osmotic pressure, buffering capacity, and redox potential (Paswan *et al.,* 2022).

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Table 1. Previous investigations of rumen virus populations, rumen microorganisms (bacteria and archaea), and viruses isolated from the rumen have proposed roles and properties of virus populations inside the rumen. Adapted from (Gilbert *et al.*, 2020)

Functions and characteristics of the viral populations in the rumen	Previous publications
-Viruses, are constantly present in large quantities and rudely reproduce. in the microbial populations of the rumen.	Adams *et al.*, 1966 ; Ritchie *et al.*, 1970.
-Viral replication and Viral proteins lyse microbial cells and liberate nucleic acids and microbial proteins, which aid in the intraruminal recycling of nutrients.	Nolan and Leng, 1972; Orpin and Munn, 1974; Wells and Russell, 1996; Solden *et al.*, 2018.
- Viruses are genetic elements on the move; Viruses allow the steady integration of virus-encoded (prophage) genes into the genomes of rumen microbial organisms and transfer genetic material across bacteria.	Klieve *et al.*, 1989; Cheong and Brooker, 1998; Attwood *et al.*, 2008b; Seshadri *et al.*, 2018.
-Virus populations generate viral particle booms and periods of microbial lysis, which in turn foster microbial diversification by predation.	Hoogenraad *et al.*, 1967; Iverson and Millis, 1977; Klieve and Swain, 1993.
- Virus infection and the emergence of resistance able to changes in bacterial growth habits, such as a rise in bacterial extracellular polysaccharide.	Klieve and Bauchop, 1991; Klieve *et al.*,1991.
- The composition of the rumen virus population is dynamic and vary in response to shifts in the number of microorganisms and their diet.	Swain *et al.*, 1996a ; Klieve *et al.*, 1998; Anderson *et al.*, 2017.
- The prokaryote virus order Caudovirales, or tail-phase viruses, is the most prevalent among the taxonomically varied group of rumen viruses.	Ritchie *et al.*, 1970 ; Klieve and Bauchop, 1988 ; Berg Miller *et al.*, 2012.
- Limited microbial host range refers to the tendency of viruses, particularly phage, to infect exclusively certain rumen bacteria.	Iverson and Millis, 1976a ; Styriak *et al.*,1989 ; Klieve *et al.*, 1999.
- Extended contact with rumen fluid can lead to the degradation of virus particles, particularly phage particles.	-Iverson and Millis, 1976a ; Smith *et al.*, 1987 ; Nemcova *et al.*, 1993.
- The development of genetic resistance mechanisms, such as Cas systems, is facilitated by virus infection.	-Berg Miller *et al.*, 2012 ; Gilbert *et al.*, 2017
- Viral interactions with rumen epithelial cells have been demonstrated in vitro.	Styriak *et al.*, 1991

The release of CH_4 from enteric fermentation and manure handling, as well as the frequently intense cycling of nitrogen (N) on dairy farms, emissions from these farms are high. This phenomenon results in both direct and indirect emissions of nitrous oxide (N_2O) from soils, as well as N_2O lost through ammonia (NH_3) volatilization and nitrate leaching (Olesen *et al.,* 2006; Maranon *et al.,* 2011). A further energy loss to the animal is methane generation, which can range from 2 to 12% of gross energy intake according to intake amount and feed mix (Sejian *et al.,* 2011). Yet, it is also conceivable that feed efficiency improvements could

decrease the quantity of feed and waste (such as manure and CH_4 emissions) generated per unit. But shortly, reducing the environmental impact of the livestock sector will be just as important as improving the feed efficiency of animals (Godfray *et al.,* 2010). In the next point, we will highlight the factors that determine higher or lower enteric methane, focusing mainly on the feed type received.

1.2.1. Influence of forage-based diets

The type of carbs in the fodder determines how easily it is absorbed. Forages are high in free sugars and starch that are simple to digest and typically have shorter rumen retention times, producing less of CH_4 than forages high in structural carbohydrates, like acid detergent fiber (ADF) and neutral detergent fiber (NDF) (Niu *et al.,* 2018). Higher proportions of structural carbohydrates in forages are associated with lower quality; these levels rise with age (Ortega-Gómez *et al.,* 2011). However, there is a wide range in the nutritional quality of fodder, depending on several characteristics such as age, fertilization, and season (Barahona and Sánchez, 2005). High lignin and fiber content lower grazing cattle's voluntary fodder intake (Richmond *et al.,* 2015).

Forages with different natures (such as fresh herbage and silage) and forage types (such as grass, legume, or maize) plus animal categories (cattle and sheep) have all had their effects on the digestibility of forage organic matter (OM) studied about CH_4 emission intensity examined. For dairy and beef cattle, but not for sheep, enhanced digestibility of the feed led to higher dry matter intake (DMI, kg/day) and, as a result, higher CH_4 emission (g/day) For intake or production, there is a drop in CH_4 yield (g/kg DMI) and CH_4 intensity (g/kg milk) for dairy cattle with more digestibility; however, there is no discernible variation in CH_4 yield (g/kg DMI) for beef cattle (**Figure 15**).

With the higher digestibility of the feed, sheep's CH_4 yield dropped (Van Gastelen *et al.,* 2019). Feeding forages, particularly legume species, is an intriguing way to reduce greenhouse gas emissions while giving animals nitrogen, improving animal productivity (growth, milk, and wool production), and reducing emissions of ammonia, CH_4, and N_2O. Studies conducted *in vitro* (Makkar, 2003a) demonstrated that forage legumes with condensed tannins, birds-foot trefoil, sulla, or polyphenol oxidase enzymes decrease the breakdown of rumen proteins, and ruminants appear to absorb these proteins into milk and meat. (Hristov *et al.,* 2013; Van Gastelen *et al.,* 2019) have compiled findings from many studies comparing the amount of CH_4 that ruminants fed maize silage instead of grass or legume silage emit. The loss of maize silage for grass silage in the diet can lower CH_4 emissions.

Methanogenesis may decrease because of grass silage's increased digestibility (Van Gastelen *et al.,* 2019). Methane production rises due to increased fiber content at the advanced stage of development. Because forage legumes increase biodiversity, reduce parasitism in ruminant populations, and reduce N fertilizer consumption and production, they can reduce greenhouse gas emissions in ruminant systems (Eugène *et al.,* 2021). Dietary changes can significantly alter the microbial makeup of the rumen, perhaps outweighing the host's influence (Purushe *et al.,* 2010).

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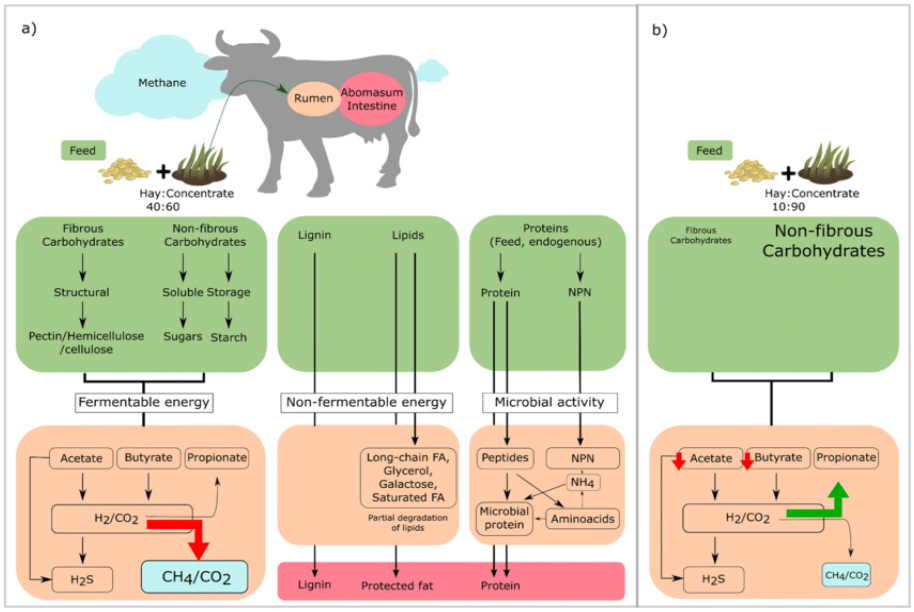

Figure 15. a) Volatile fatty acids (VFAs), hydrogen (H_2), and carbon dioxide (CO_2) are produced during the fermentation process during ruminant digestion (NPN = nonprotein nitrogen). b) A greater proportion of carbohydrates that ferment more quickly lowers the levels of acetate and butyrate and raises propionate, which in turn lowers the amount of methane (CH_4). Adapted from (McCauley *et al.*, 2020).

According to Henderson *et al.* (2015), a global rumen census effort using many geographically diverse rumen samples, it was found that, regardless of the ruminant species, a forage-based diet results in a higher abundance of the bacterial order *Bacteroidales* and family *Ruminococcaceae,* whereas a grain-based diet results in a higher abundance of the genus *Prevotella* and family *Succinivibrionaceae*. The amount and composition of the cow's feed (cellulose, hemicellulose, and non-fiber carbohydrates) in terms of fiber are used to compute the synthesis of CH_4 (Castelán-Ortega *et al.,* 2014). The efficiency of fermentative digestion in the rumen and the efficiency of feed conversion to product (such as milk or beef), which in turn depends on the balance of nutrients absorbed, are the two factors that determine methane output relative to the product output of ruminants fed low-quality forages (Bhatta *et al.,* 2017). The effectiveness of modifying fodder through physical processes like grinding and pelleting, as well as chemical processing like sodium hydroxide and ammonia, to increase its digestibility in the rumen (Adesogan *et al.,* 2019).

Carbohydrates have less of an impact on CH4 production at low intake than they do at greater intake (Shibata and Terada, 2010). For instance, in a large-scale dairy farming system, feeding premium lucerne and corn silage can reduce methane output by 10% -20% (Mitsumori and Sun, 2008). Seasonal fluctuations in pasture methane production; hence, methane emissions from grazing ruminants are affected by seasonal differences (Lovett *et al.,* 2006). Changes in the nutritional value of forage should reduce methane generation by 20-40%, as predicted (Mitsumori and Sun, 2008). Other strategies for methane mitigation through nutrition include lowering the ratio of concentrate to forage in diets, adding more oil to the diet, and including methane inhibitors and rumen modifiers (Haque, 2018).

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1.2.2. Influence of grain-based diets

Milking cows frequently eat cereal grains, but little study has been performed on how feeding wheat affects the methane emissions from the cows' enteric digestion (Moate *et al.,* 2016). When cows fed byproduct concentrate were compared to concentrate manufactured from grains, there were signs of improved energy status based on blood parameter readings. It may account for the production level maintained regardless of the source of dietary concentrate **(Figure 16)** (Pang *et al.,* 2018). The quality of food and its constituent parts have tremendous effects on CH_4 production. The primary source of cow energy loss is this, so minimizing it is essential to raising average daily gain (ADG) or milk production. As a percentage of the GEI or DMI (kg/d), high-quality grain-based diets supply more energy for animal production and reduce maintenance costs more than low-quality forage-based diets or grazing, which lowers the CH_4 g/kg ECM (Bačėninaitė *et al.,* 2022).

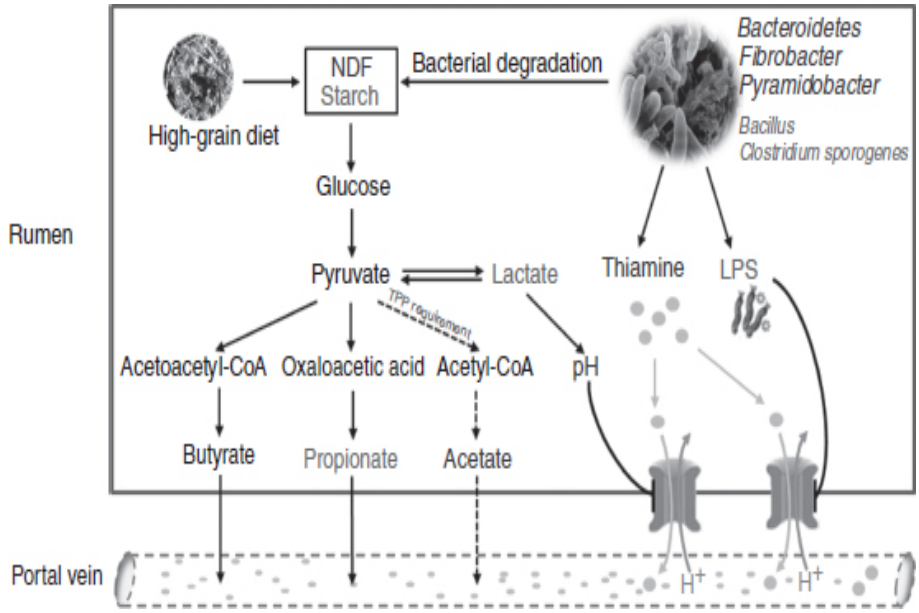

Figure 16. A high-grain diet causes less neutral-detergent fiber (NDF) and more starch to enter the rumen of dairy cows. Then, pyruvate is used by bacteria to break down carbs into volatile fatty acids (VFA). Pyruvate builds up during this process, and more thiamine is required to convert it to acetyl-CoA in the form of the cofactor thiamine pyrophosphate (TPP). Thiamine concentrations drop as a result, and more pyruvate flows to lactate and propionate, lowering pH. Adapted from (Pan *et al.*, 2018).

Raising the starch level in diets containing moderate amounts of grains changes the taxonomic composition and decreases the diversity and richness of the rumen microbiota in cattle (Khafipour *et al.,* 2016). From 0% to 50% of DM in the diet, the DMI went from 11.4 to 15.6 kg/day. Along with lowering the rumen pH (Pang *et al*., 2018, Figure 18) and increasing the concentration of total VFA and the molar proportion of butyrate in the rumen digest, this increase also decreased the acetate-to-propionate ratio and the molar percentage of acetate (Plaizier *et al.,* 2018; Kotz *et al.,* 2020; Hua *et al.,* 2022). Starch-fermenting bacteria in the rumen can readily break down cereal grains, which have a far higher amount of stored carbohydrates than grass. Elevated concentrate content in the feed reduces methane emissions (Mitsumori and Sun, 2008).

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Rumen microbes quickly ferment the starch in cereal grains. Since the diet contains a lot of concentrates and 23% of propionate is made from pyruvate via the acryl CoA pathway (the acrylate pathway), it is assumed that some bacteria that break down starches produce lactate, which is then used by *Megasphaera elsdenii* to produce propionate (Prabhu and Eiteman, 2012).

1.2.3. Ruminal pH regulation

The pH of the rumen varies between 5.5 and 7.0 based on the type of feed. The ideal temperature for rumen microbes to provide efficient fermentation is between 38 and 40°C, as stated by Öztürk and Gursel. (2021). Ruminal pH is the product of the host and the rumen working together to regulate acid-base balance (including the rumen bacteria) (Humer *et al.,* 2018). Since the growth rates and fermentative abilities of the principal bacteria that produce lactic acid, such as *Streptococcus bovis*, and the bacteria that use it, like *Megasphaera elsdenii*, are in balance, the production of lactic acid is quick. Hence, relative to lactic acid, an increase in total VFA causes a decrease in rumen pH within this field (Goad *et al.,* 1998; Enemark *et al.,* 2002). Rumen digestion impacts the rumen pH, which defines the type of digestion that occurs in the rumen.

Most rumen bacteria grow faster at first when the amount of readily fermentable carbohydrates in the diet increases (Nocek and Russell, 1988). Long-term low pH levels in the rumen prevent cellulolytic bacteria from adhering to cellulose (Mourin *et al.,* 2001). It can lead to a range of health issues for the animal, including rumen ulcers, liver abscesses, founder, and even death (Russell *et al.,* 2009). Though Gram-negative bacteria also proliferate in extreme cases of rumen acidity, these conditions lead to a decrease in the number of cellulolytic bacteria and a change in the bacterial population, such that Gram-positive cocci and rods become the majority (Nagaraja *et al.,* 1978; Goad *et al.,* 1998).

Data on ruminal bacterial species alterations brought on by rumen pH fluctuations are scarce. One explanation for this is the lack of knowledge regarding the variables affecting rumen bacterial development *in vivo*, such as nutrient and environmental needs, pH, and oxygen availability in rumen (**Figure 17)**. *Entodiniomorphid* protozoa, among other protozoal species, are crucial for preserving the pH of the rumen. This is so that bacteria cannot quickly digest starch by creating lactic acid, as these protozoa absorb starch and sequester it from bacteria (Bonhomme, 1990). Instead of being used for cell development in low-pH environments, energy is used for non-growth functions, such as preserving the intracellular pH at an ideal level (Oba and Allen, 2003). A direct correlation between microbiological efficiency and pH levels. Low pH is speculated to prevent ruminant microbial efficiency by impeding digestion (Bünemann *et al.,* 2020).

1.3 NUTRIENT REQUIREMENTS OF DAIRY CATTLE

Feeding dairy cattle utilizes the sciences of nutrition, biochemistry, and microbiology and combines them with animal husbandry. Diet formulation for the high-producing dairy cow requires knowledge of the nutrients needed by the mammary gland to produce milk. These nutrients include water, protein (amino acids), carbohydrates, fats, minerals, and vitamins (Erickson and Kalscheur, 2020). Understanding their physical characteristics and their combined interactions is essential for the successful feeding of dairy cattle (Erickson and Kalscheur, 2020).

1.3.1 Water

Milk is 87% water and is essential for milk production. According to Murphy (1992), water comprises between 56% and 81% of a dairy cow's total body weight and between 68% and 72% of ruminant calves (Chapman *et al.,* 2017). Water is necessary for the body to move materials around, regulate body temperature, maintain insulation, and eliminate waste. As previously mentioned, water is crucial for the emergence of rumen microbiota in pre-ruminant calves and is eventually required for the calf's transition from non-ruminant to ruminant. There are three types of water sources: feed water, endogenous reaction water, and free water (drinking water). Free water intake is the main supply of water for livestock. Consequently, anything that hinders an animal's access to free water will impair its performance. Free water intake rises in proportion to an increase in milk production, as would be predicted. When a cow is in an environment with a temperature-humidity index higher than 68, its daily consumption of water also increases. Murphy *et al.* (1983) equation states that the free water intake ranges from 92 to 138 L/d. This formula takes into consideration the minimum temperature, milk yield, sodium intake, and feed intake. Two equations that currently appear to be appropriate to employ when calculating free water consumption for cows were obtained by Appuhamy *et al*. (2016).

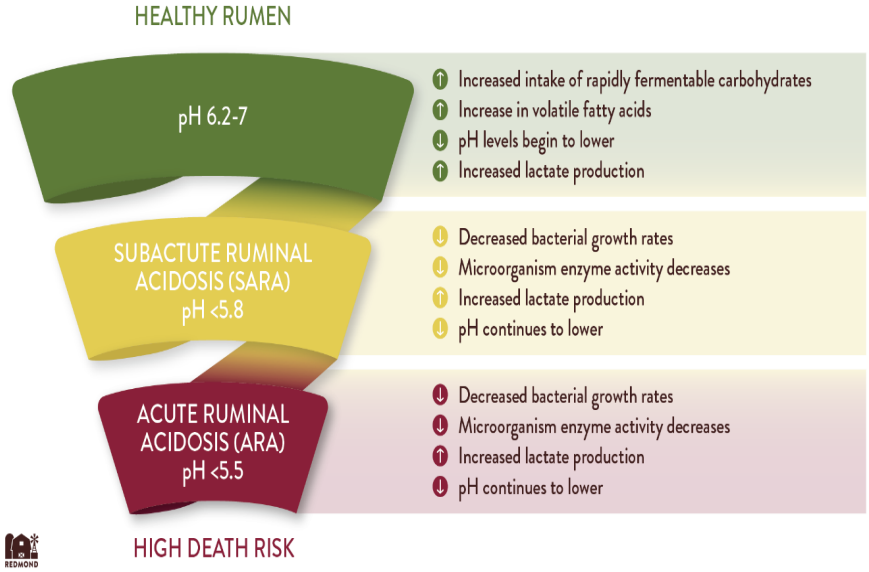

Figure 17. Reflects the modifications to the ruminal environment carried through a pH shift. (Source: https://blog.redmondagriculture.com/correcting-ruminal-acidosis, 2024).

1.3.2. Proteins and amino acids

Cow's milk comprises about 25 distinct proteins, only a minority of which are allergic. Each litter contains about 30-35 g of proteins. Crude protein (CP) is the unit of measurement for protein in feedstuffs and is calculated by multiplying the percentage N in a feed by 6.25. The feed proteins' roughly 16% N content is the source of the value of 6.25. But in addition to actual protein, crude protein also contains other nitrogen-containing substances such as amino acids, dipeptides, nucleic acids, NH_3eN, and other non-protein nitrogen (NPN) components. Early on, it had to be recognized that, like other animals, cattle have an amino acid requirement

rather than a protein requirement, with actual proteins being defined as chains of amino acids (Erickson and Kalscheur, 2020).

1.3.3. Carbohydrates

Nutrients derived from carbon, hydrogen, and oxygen are called carbohydrates. Carbohydrates contain more oxygen molecules than fats. Additionally, they make up most of the dairy cattle's diet. Carbohydrates were classified as chains of simple glucose. As with other ruminants, the two most common carbohydrates fed to dairy cattle are cellulose (1,4 linked glucose units) and starch (amylopectin and 1,4 and 1,4 linked glucose units). Carbohydrates can be identified as grains, sugars, roughages, and forages. Cellulolytic bacteria are principally responsible for the digestion of forages such as hay, hay-crop silage, and grain-based silage made from maize or small grains. According to National Research Council (NRC, 2001), this process produces acetic and butyric acids.

1.3.4. Fat

With 2.25 times the energy of protein or carbs, fat is the most energy-dense nutrient. Uniquely in cases where cattle are under heat stress, fat can help sustain caloric intake because it is not significantly digested in the rumen, producing little heat of fermentation. kinds of fat Glycerol and non-glycerol are the two categories of fat. Glycerol-type fats, which comprise triglycerides, phospholipids, and glycolipids, are of nutritional benefit, whereas non-glycerol-type fats, which include waxes and sterols, have little to no nutritional value (Beede and Washburn, 2017). These fats have elongated carbon chains with the phosphate, glucose, or glycerol moiety as their main constituent. Rumen microorganisms can change the normal even number of carbon fatty acids into odd C-chain fatty acids by incorporating fatty acids into their cell membrane. Saturated fats are fully hydrogenated, while unsaturated fats contain double bonds. While most fats from terrestrial animals include some level of saturated fat, most fats from plants are unsaturated (Erickson and Kalscheur, 2020).

1.3.5. Minerals

Minerals are categorized as metal elements, which are inorganic substances needed for a wide range of physiological processes, including osmotic equilibrium, nerve impulses, and structure. Certain minerals (like glutathione peroxidase) are required for the activity of enzymes or act as catalysts for processes. Minerals are separated into two groups. 1) Macrominerals, which are needed in grams and include Ca, P, Mg, K, Cl, Na, and S. 2) Microminerals, sometimes referred to as trace minerals, are needed in mg or mg amounts. Mineral nutrition is critical to the lactation performance of dairy cattle. There is a significant pull-on due to the amount of milk that cows produce upon parturition. This condition frequently results in hypocalcemia in the cow, further referred to as milk fever or parturient paresis. The cow will be unable to stand, have chilly ears, and frequently have a low body temperature in this condition (Erickson and Kalscheur, 2020).

1.3.6. Vitamins

Vitamins are organic substances categorized as lipid-soluble and water-soluble. Vitamins that dissolve in an aqueous solution are known as water-soluble vitamins. In the rumen, they are synthesized in sufficient amounts. Nonetheless, some water-soluble vitamins can be

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advantageous when taken as supplements. Vitamins that are lipid-based are known as lipid-soluble vitamins. These are K, E, D, and A vitamins. Adequate supplies of vitamin K are synthesized by the rumen microorganisms. Depending on the diet, distinct amounts of supplements for vitamins A, D, and E are required (Erickson and Kalscheur, 2020).

1.3.7. Feed Additives

Dairy cattle diets typically encompass feed additives to enhance many performance metrics, including growth, feed efficiency, milk component output, growth, and health. Their application ought to be predicated on an objective study. The popular feed additives, like ionophores, probiotics, saponins, and essential oils (Eickson and Kalscheur, 2020).

1.4. DISEASES ASSOCIATED WITH MICROBIOTA DISORDERS IN COWS' HEALTH

Through enteric fermentation, methane production can result in losses of up to 12% of the gross energy intake (Johnson and Johnson, 1995). Feeding techniques, feed supplements, and selective breeding represent a few ways in which livestock might lower their CH_4 emissions (Kumari *et al.,* 2020; Reisinger *et al.,* 2021). As reported by Shabat *et al.* (2016), there has been a correlation between the relative abundance of *Methanobrevibacter ruminantium* species and *Megasphaera elsdenii* species in Holstein cattle that are related to lower feed efficiency and estimated methanogenesis potential. Based on Ross *et al*. (2012), ruminants can transform fibrous, low-quality plants into high-quality milk and meat. The kind, quality, and quantity of feed that cattle receive are considerable factors that determine how much enteric CH_4 they produce (Hatew *et al*., 2015).

The proportion of fat, protein, carbs, and minerals in the forage determines its quality. Because it significantly affects the dairy industry's profitability and environmental efficiency, enteric fermentation is a valuable quality in real-world situations. Increased DMI was explained by improved digestibility and decreased CH_4 generation (Guinguina *et al*., 2020). Higher milk production raises the energy of cows, which causes the body to mobilize layers of tissue to meet the increased energy demands of lactation (Connor, 2015). The generation of CH_4 and digestibility have a positive interaction (Løvendahl *et al*., 2018; Guinguina *et al*., 2020). DMI has a greater impact on cow efficiency differences than comparable changes in BW and milk yield (Potts *et al*., 2015; Xi *et al*., 2016).

Beef and dairy products are examples of high-quality human-edible food that ruminants may produce from human-inedible feed, such as grasses, forages, and by-products high in cellulose. Compared to other livestock species, ruminants have a minimal conversion efficiency, particularly when it comes to growing cattle. This minimal conversion efficiency corresponds to greenhouse gas emissions and nitrogen pollution (Cantalapiedra-Hijar *et al*., 2018). The potential of an animal to modify ingested feed into nutrients available for metabolism determines feed efficiency. It was widely assumed that various digestibility was the same. Nevertheless, each cow has distinct characteristics regarding how effectively animals can digest certain feedstuffs (Huhtanen *et al*., 2015; Cabezas-Garcia *et al*., 2017).

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Extensive microbiomes observed in the digestive tracts of dairy cows serve to digest high-fiber feed and emit CH_4 consequently. One of the most prevalent feed efficiency metrics in

research settings is residual feed intake (RFI) (Connor *et al*., 2019; Flay *et al*., 2019; Shonka-Martin *et al*., 2019). As output and feed intake rise, more and more of the energy intake from the feed is applied to producing milk rather than for maintenance purposes. Elite dairy cows today yield more than 4× their energy needs for maintenance in the form of milk and meat (VandeHaar and St-Pierre, 2006). The digestibility and metabolizable of the food are favorably correlated with milk energy output adjusted for zero energy balance (EB) per unit of GEI, but CH_4 energy as a percentage of GEI at the same DMI/body weight (BW) was negatively correlated.

When selecting against CH_4 emission in breeding programs, it should be performed to avoid the risk of selecting for reduced digestibility, which is a crucial component of ruminant nutrition, if there is a relationship between cow variation in CH_4 yield and variations in the ability of cows to digest a particular diet. Conversely, a beneficial strategy to lower CH_4 emissions is to produce cows with high lactation efficiency of metabolisable energy (ME) (Guinguina *et al*., 2020). Improvements to the feed quality substantially reduce the amount of land and other resources required to make nutrition and help prevent undesired greenhouse gas emissions (Løvendahl *et al.,* 2018). Furthermore, as per Hardison *et al*. (2017), dairy producers may experience financial gains by reducing their feed consumption. Furthermore, the loss of balance between the different components of the rumen flora, described in a previous section, apart from the effects on GHG emissions, may have effects on the health status of the animal. The main diseases associated with this are described below:

1.4.1. Bloat

The state identified as rumen tympany, or bloat, occurs due to an accumulation of extra gas in the rumen. It appears in animals fed larger amounts of forage or grains (Kaba *et al.,* 2018). It is classified as frothy bloat and free gas. Free-gas bloat is a pathological or physical issue that prevents the dairy cow's stomach from releasing gas. Feed intake causes frothy bloating because it continuously builds froth that is difficult to pass from the stomach.

Hydrophobic and hydrophilic characteristics have been found in foamy bloat. Polymeric substances such as fatty acids, glycans, glycolipids, and lipopolysaccharides partially break down to produce foam. These partially digested compounds make it harder to extract gas from the rumen. Organ failure, edema, agony, and death can result from gaseous distension's pressure on the surrounding organs. In addition to physical elements, the rumen bacteria play a role in the onset of gas (Xu *et al.,* 2021).

Methanogenic bacteria that feed on a variety of substrates produce gas. An accumulation of gas in the stomach is caused by excess methane, hydrogen, and CO_2 gases are produced when downstream microorganisms ignore them. In addition, the hydrogen gas generated during the metabolism of methanogen needs to be handled. The rumen environment is known to be quite anaerobic.

However, too much CO_2 might subtly affect the rumen. Methanogens can convert CO_2 to produce methane, and as a result, more CO_2 can cause tympany (Trefz et al., 2017). Nonetheless, to preserve the balance of the ruminal microbiota, it is imperative to address the overproduction of these gases. Probiotics are utilized to restore the rumen flora and maintain the environment. Methanogenic bacterial stress can also be reduced with treated and high-fiber feed (Dougherty, 2009).

1.4.2. Ruminal acidosis

More fermentable feed material rich in carbohydrates is consumed than grainy feeds, which results in ruminal acidity (Nagaraja and Titgemeyer 2011; Hernández *et al.,* 2014). Acidosis occurs in potatoes, sugar beets, molasses, and cereal grains. The rumen's pH is significantly lowered as a result of the fermentation of these substances, which increases the formation of lactic acid (Abdela, 2016; Jaramillo-López *et al.,* 2017).

The data of several gram-negative bacteria, release endotoxin into the rumen. Low pH, fluid buildup, disruption of the microbiota, and incomplete digestion are the outcomes of all these. Acid production and low pH had detrimental effects on the stomach's inner epithelium, including ulcers and inflammation. A sharp drop in pH also suppresses the rumen's propionate-producing bacteria while boosting the cellulolytic bacteria. According to Plaizier *et al.* (2008), changes in the rumen microbiota can result in abnormal metabolism, which can induce lung-related illnesses, liver malfunction, and even mortality.

1.4.3. Reticuloperitonitis

Cattle with erratic feeding practices are susceptible to reticuloperitonitis, often known as traumatic reticulitis or hardware disease (Braun *et al.,* 2020). The balance of the rumen microbiota is upset by the dairy cow's indiscriminate feeding practices. Frequent feeding inhibits the resurgence of bacteria and leads to poor digestion, potentially resulting in gas and acidity in the rumen. This disease is not infectious, but if left untreated, it can have disastrous consequences. Bacterial resurgence is facilitated by consuming the right food at regular times. According to Braun *et al.* (2018), rumenotomy is prescribed during the severity index, and the use of probiotic syrups, antibiotics, and digestive aids may be helpful in the beginning.

1.4.4. Diarrhea

In young calves, diarrhea is a severe problem that frequently ensues. It caused several symptoms, such as weakness, dehydration, and disruption of electrolyte balance. The disease's cause varies depending on the host's metabolic problems, the type of feed, the location, and the infection. Bacteria (*Salmonella sp., Clostridium perfringens., Staphylococcus., and Mycobacterium paratuberculosis*), viruses (*rotavirus and adenovirus*), parasites, and protozoa (*Strongylus sp., Trichonema sp., Entamoeba sp., and Giardia sp*.) are pathogens that induce diarrhea (Xu *et al.,* 2021). If any of the organisms above infect the ruminant flora, it disrupts its homogeneity and functionality, which leads to illness (Bhattacharjya *et al.,* 2018).

1.5. VETERINARY STUDIES CARRIED OUT TO MINIMIZE METHANE EMISSIONS

A transnational study is demanded to estimate the amount, the sources, and the trails of emissions from diverse supply chains and animal exhibition approaches. By using these details, specific guidance for reducing emissions might be created (Gerber *et al.,* 2013; Castillo, 2018). In addition, there are numerous ways to lessen the amount of CH_4 that operations emit. To quantify the effects of physiological stage and food features, lactating or nonlactating state, local conditions, and accessible fodder, Ricci *et al.* (2013) conducted a systematic analysis of methane emissions from beef and dairy. Terry *et al.* (2020) stated that food is critical for decreasing CH_4 emissions. To achieve the 1.5°C target, ruminant CH_4 emissions must decrease

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from 2010 levels by 11–30% by 2030 and 24– 47% by 2050 (Arndt *et al.,* 2022). Utilizing feed additives and methanogenesis inhibitors is one way to lower methane results during digestion. Methane inhibitors work by changing the structure or function of primary molecules that promote the release of methane.

These feed additives perform by either blocking methanogenesis or changing fermentation to encourage alternate H_2 uptake as propionate synthesis (McAllister and Newbold, 2008; Martin *et al.,* 2010). Some strategies can lower ruminant enteric methane emissions by 12% to 32%, either absolute or relative per unit (Arndt *et al.,* 2022). Improving pasture quality (i.e., digestibility) and adding additional concentrate feeds to the diet—two of the three so-called product-based mitigation strategies—might be relevant to the US dairy business. To minimise greenhouse gas emissions from ruminant agriculture without sacrificing animal production, researchers are looking for natural substitutes to include in the diets of grazing cattle (Tedeschi *et al.,* 2021).

Finding feed additives that boost output, or lower methane emissions is in steadily increasing demand (Belanche *et al.,* 2020). The most substantial source of methane emissions globally is cattle ranching. Methane emissions and global temperatures rise in concert with the growing demand for high-quality meat and dairy products. Therefore, reducing ruminant methane emissions is one of the best ways to mitigate the effects of climate change. The most economical method is still feed manipulation, which may provide a significant 60% reduction in methane by carefully choosing the right kind or quality of forage and adjusting the feed's concentrate-to-forage ratio. A great deal of potential exists for both organic and inorganic feed additives to reduce the production of CH_4 by altering the rumen microbial community, directly or indirectly. *In vitro* and *in vivo*, rumen methanogenesis has decreased due to chemical additives such as 3-nitroxypropanol (3-NOP), ionophores, and halogenated compounds. These additives work as direct inhibitors of methanogens or by promoting the growth of bacteria that compete with methanogens for the same substrate (Tseten *et al.,* 2022).

1.5.1 Microflora modifying inhibitors

Microflora-modifying inhibitors (MMIs) include tannins, saponins, and monensin (MON). Their general effect is the reduction of substrates for methanogenesis because they can inhibit protozoa and gram-positive bacteria (Hook *et al.,* 2010). All methane inhibitors fall into one of three categories: microflora modifying inhibitors (MMIs), enzymatic inhibitors (EIs), and hydrogen receptors (HRs), according to their distinct ways of inhibiting methanogenesis.

1.5.1.1 Monensin

Rumensin, also known as monensin, enhances feed digestibility, changes the volatile fatty acid (VFA) profile from acetate to propionate, lowers methanogenesis, lowers ruminal ammonia concentration and amino-acid fermentation boosts hepatic gluconeogenesis, and increases the animal's energy supply (Azzaz *et al.,* 2015). Around the world, poultry and livestock breeders have depended on the polyether ionophore antibiotic monensin as a coccidiostatic and growth-promoting drug (Chapman *et al.,* 2010). Earlier comprehended as monensic acid, monensin is a monovalent carboxylic ionophore polyether antibiotic made by *S. cinnamonensis* (**Figure 18**).

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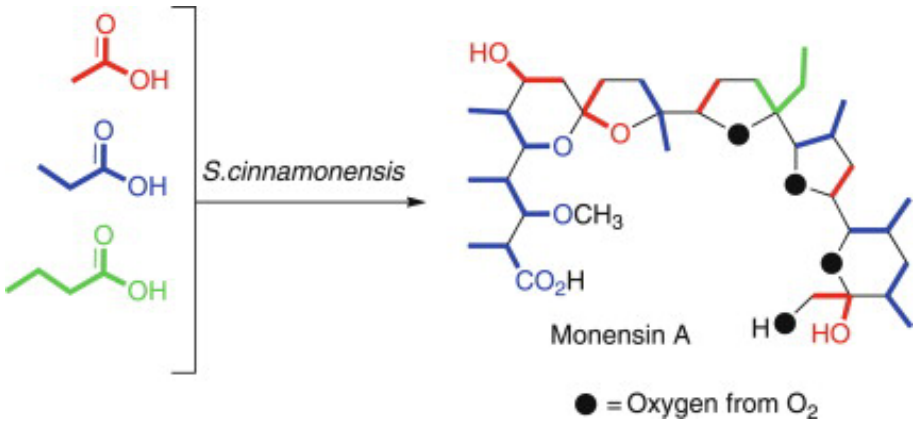

Figure 18. Origin of the carbon and oxygen atoms in monensin. Adapted from (Hill and Stauton, 2010).

More effectively than K^+, it transfers Na^+ (Samanidou and Evaggelopoulou, 2008). As reported by Gupta *et al.* (2019), developing heifers that received a monensin supplement (approximately 0.6 mg/kg, BW) produced less enteric methane. Chemical substances known as ionophores, or ion carriers, are included in diets. Although some ionophores, such as lasalocid, lincomycin propionate, and monensin, are available in the United States, monensin is the most frequently utilized (Tedeschi *et al.,* 2003). Since its discovery in 1969, monensin has been used as a coccidiostat in livestock worldwide. The molecule's capacity to diminish Gram-positive bacteria in the rumen while increasing Gram-negative bacteria enhances energy metabolic efficiency, which has several positive effects on animal health and productivity (Duffield and Bagg, 2000).

The chemical improves feed efficiency by changing rumen fermentation to produce more propionate while reducing carbon loss as carbon dioxide and methane. Monensin's method of action has been the driving force behind its usage as a feed addition in cattle nutrition for many years (Russell and Strobel, 1989). Monensin primarily affects the rumen microbiota by altering the cell bacterium's sodium-potassium balance. Consequently, the bacteria use more energy to restore equilibrium, which inhibits both growth and death. The ruminal bacterial population shifts by this event, but not exclusively, impacts Gram-positive bacteria and gives them an edge over Gram-negative bacteria (Schären *et al.,* 2017).

Given that propionate is the most effective energy source for ruminants during ruminal fermentation, there is an increase in the engagement of Gram-negative bacteria, such as lactate consumers and propionate makers, in the rumen. Parallel to this, the rumen experiences a decline in the acetate: propionate relation, which raises the metabolizable energy because of a drop in acetate synthesis (Prado *et al.,* 2010). Due to a decline in proteolytic and fermentative amino acid bacteria, which support protein metabolism, there is more protein from food available in the small intestine (Rangel *et al.,* 2008). Monensin, an antibiotic, also has antifungal and antiprotozoal (anticoccidial) qualities. It is frequently used as a coccidiostat to promote growth in livestock, such as pigs, poultry, and cattle (Mimouni *et al.,* 2014). In commercial feedlot cattle diets, monensin is implemented to control ruminal fermentation to increase feed efficiency and weight gain (Ipharraguerre and Clark, 2003). It demonstrated that adding monensin to the diet lowers the production of ammonia, enteric methane, and ruminal acetate

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to propionate ratio while increasing feed efficiency and daily weight gain (Hemphill *et al.,* 2018).

By focusing on inhibiting gram-positive bacteria, monensin improves nitrogen metabolism by reducing the formation of ammonia and lactate and increasing the efficiency of propionate production in energy metabolism (McGuffey *et al.,* 2001). Depending on the diet's forage-to-concentrate ratio, amount of supplemented monensin, and composition, monensin feeding is frequently linked to depression in DMI (Aderinboye *et al.,* 2012). According to research conducted by Duffield *et al.* (2008), monensin is known to lower the percentage of milk fat without affecting the overall output, but it does not influence protein or casein contents. Research conducted in vitro has shown that monensin decreases the ability for biohydrogenation (BH), and that obtained in vivo indicates that the effects of monensin on milk fat when combined with a high rumen unsaturated fatty acid (UFA) load or increased dietary starch (He *et al.,* 2012). When considered, supplementing with monensin improved component-corrected milk production efficiency and estimated diet energy content linearly, but it had no change in milk fat (Horst *et al.,* 2023).

Monensin is a common feed additive used to increase ruminant feed efficiency; however, the benefit of this ionophore is prohibited in many countries, like European Union due to the antimicrobial resistance in humang beings, and substitutes have been researched (Wanapat *et al.,* 2015).

The widespread use of monensin in cattle and poultry systems, along with the possibility of indirect human exposure and its toxicity profile, make it a high-priority pollutant of rising concern in water supplies (Capleton *et al.,* 2006; Pérez *et al.,* 2021). Antibiotic growth promoters, or AGPs, have been illegal in Europe since 2006 (Cogliani *et al.,* 2011); however, several other nations, including the USA, China, Japan, Australia, Brazil, and Argentina, continue to use them regularly (Kumar *et al.,* 2012; Maron *et al.,* 2013).

By establishing multiple laws and directives, the European Union (EU) has tightly regulated controls on the use of veterinary medications, including growth-promoting substances, especially in species of food animals (Mezzetti *et al.,* 2019). The EU decided to stop marketing and using antibiotics as growth promoters in feed due to concerns about bacterial resistance. 1997–1999 saw the withdrawal of several antimicrobials, including avoparcin, ardacin, vacatitracin zinc, virginiamycin, tylosin, or spiramycin. Four more substances, flavophospholipol, avilamycin, salinomycin sodium, and monensin sodium, were still used as growth promoters in animal feed for sale in the EU. They have also been prohibited since January 2006 (Samanidou and Evaggelopoulou, 2008).

Monensin's impact on DMI has been a topic of argument for many years. When cows are in a positive energy balance, the increased propionate production caused by monensin affects satiety, which lowers DMI (Allen, 2014). Contrarily, because propionate production is not at the levels that promote satiety, results from the transition phase confirm that this effect does not exist in the early stages of breastfeeding. It is believed the transition phase, food digestibility, and rumen fill would have more influence on feed consumption than propionate supply (Allen, 2020). The elevated plasma level of urea in response to monensin by controlled release capsule treatment is an additional intriguing finding (Duffield *et al.,* 2003; Kasap *et al.,* 2020). Numerous authors reported this outcome, which they linked to an increase in the flow of undigested proteins into the gut, where they were able to escape the rumen because there were

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fewer protein-degrading bacteria than used the absorbed AA for gluconeogenesis (Duffield *et al.,* 1998).

More evidence for this theory came from the higher urea content of milk (McCarthy *et al.,* 2015) and the lower ammonia levels in the rumen fluid of cows treated with CRC (Mezzetti *et al.,* 2019). There is also a concept that suggests that the medication leads to an increase in urea synthesis in the liver because hepatocytes accumulate less fat, which enhances the liver's functionality. The effects of the treatment and metabolism of animals have been studied over the past twenty years. Compared to other areas, this one has more consistent outcomes. The main conclusions support the anti-ketotic benefits of monensin when given during the transition phase. The treated cows' improved energy status and liver function are primarily responsible for the decreased prevalence of peripartum illness. Research on rumen fermentation in cows receiving transition treatment has consistently shown a rise in propionate production; however, effects on ammonia production and rumen microbiota are less pronounced than those shown in cows receiving feed additive monensin (Mammi *et al.,* 2021).

Some researchers have recommended using herbal supplements, one of which is supplementing with garlic peel, as the intake of monensin may leave residues in meat and dairy products. Garlic peel and organic minerals also reduced the digestibility of crude protein. Therefore, garlic peel supplementation can be taken instead of monensin in the dairy cow diet (Prayitno *et al.,* 2019). Microbial adaptability makes the incorporation of monensin in dairy diets a less consistent methane abatement method (Almeida *et al.,* 2021). While many creative and original approaches to lowering CH_4 emissions are limited because of their effectiveness, viability, and economic cost, these are implemented on farms. In juxtaposition with employing a single or individual technique, it suggests that combining multiple measures to reduce CH_4 production at the farm level will dramatically lower the rate of CH_4 release from cows. Consequently, farmers will probably adopt CH_4 reduction measures more effortlessly if they demonstrate both nutritional and environmental benefits. For instance, adding more concentrate, fat, and oil supplements can increase animal output while lowering CH_4 production.

1.5.1.2 Tannins and saponins

When relied on as ruminant feed, forage legumes high in tannins can contain up to 20% of their dry matter in condensed tannins. Tropical forages differ from temperate forages in their chemical and structural composition and have lower digestion. Tannins have demonstrated beneficial and detrimental effects in inhibiting unwanted bacteria and protozoa (Hartinger *et al.,* 2018). But those microorganisms can also, most likely, adapt by changing their cell walls. Tannins can also lessen the amount of protein that breaks down in the rumen, this advantage is diminished if they also make the rumen undergradable protein (RUP) less digestible in the intestinal tract.

According to Jayanegara *et al.* (2012), hydrolyzable and condensed tannins also exhibit potential for reducing CH_4 emissions. Tannins can directly or indirectly reduce the rumen's release of methane by blocking protozoa or methanogens (Ku-Vera *et al.,* 2020). The kind, source, and molecular weight of tannins in the animal's methanogenic community can affect the methane response to tannin feeding. It proved that additional tropical legumes, like *Desmanthus spp.,* further reduced intestinal CH_4 emissions in cattle grazing in tropical grasslands (Suybeng *et al.,* 2019). Tannins as tanniferous plants or feed supplements have

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frequently, though not all times, demonstrated the ability to lower enteric CH_4 emissions, sometimes by as much as 20% (Beauchemin *et al.,* 2007), Studies have varied results regarding how tannins affect animal productivity and digestion.

The tannins' type, concentration, and ability to bind proteins, as well as the method used to determine the concentration of the tannins and the inability to differentiate between hydrolyzable and condensed tannins, could account for a portion of the difference (Makkar, 2003b). The danger of reduced rumen function and animal output has risen more with tannins than with saponins, according to a comprehensive evaluation of the effects of saponins and tannins on CH_4 release in ruminants (Goel and Makkar, 2012), primarily based on in vivo investigations. Thus, it may be possible to lower intestinal CH_4 output using hydrolyzable and condensed tannins, although animal production and intake may be affected. There has been conflicting research on the benefits of saponins and tannins (Almeida *et al.,* 2021; Honan *et al.,* 2021).

Studies have examined the nutritional benefits of forages high in secondary plant chemicals, such as tannins, on animal productivity and their potential to prevent methanogen development. (Jayanegara *et al.,* 2015). Adding saponin-rich sources decreased ruminal methane emission, decreased acetate proportion, and increased propionate, according to research on the effects of saponin-rich sources on methane production and ruminal fermentation parameters conducted in vitro (Jayanegara *et al.,* 2014). In their study, (Hartinger *et al.,* 2018) observed that feeding saponins can result in varying reactions (such as inhibiting protozoa) and that occasionally, there is also a drop in DMI.

Protozoa in the rumen can be reduced or eliminated by using plants that contain saponins, an approach that is becoming more and more common. Wanapat *et al.,* 2013 recommended that lower numbers of ruminal ciliate protozoa could hasten the flow of microbial proteins out of the rumen, improving feed utilization efficiency and lowering methanogenesis. most saponin sources, including *Yucca schidigera*, *Gypsophilla paniculata*, *Tribulus terrestris*, *Camellia sineis*, and *Quillaja saponaria*, have been studied (Feng *et al.,* 2012; Seradj *et al.,* 2014). In their review, (Jayanegara *et al.,* 2012) determined that the quantity of CH_4 released per unit of substrate incubated with a curvilinear pattern decreased as the concentration of a saponin-rich source increased. The study demonstrated that saponin-rich sources did not affect reducing the relevant CH_4 parameter when given at a dose of around 500 mg/g DM. At increased saponin levels, the protozoal count considerably decreased. Comparing many saponin-rich sources revealed that each of the yucca, tea, and quillaja saponins produced less methane per unit of total gas than the control ($P< 0.05$) (Jayanegara *et al.,* 2012). Although tea saponins have some promise, more thorough research is necessary before using them is advised (**Figure 20)** (Hristov *et al.*, 2013; Ku-Vera et al., 2020).

1.5.1.3. Probiotics (Yeast)

Probiotics can successfully occupy the rumen or remain while modifying the metabolic efficiency and enzyme activity of ruminant production. These comprise lactic acid bacteria, acetic acid bacteria, propionic acid bacteria, and yeasts (Sun *et al.,* 2023). To enhance the anaerobic condition, yeasts can deplete oxygen in the rumen. Yeasts can only maintain metabolic activity for roughly 30 hours because they need to settle in the rumen. Among the most prevalent safe and beneficial yeasts are *Saccharomyces, Cerevisiae, and Candida untilis*. They could continue to perform metabolically at pH 3 and exhibited a high tolerance to the

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rumen environment. Aside from competing with lactic acid-producing bacteria during the breakdown of hexose and pentose, yeasts can also help bacteria better use the hydrogen they produce. As a result, they can reduce methanogens' efficiency when using hydrogen. However, dairy cows and beef cattle have different effects of yeasts in lowering CH_4 production.

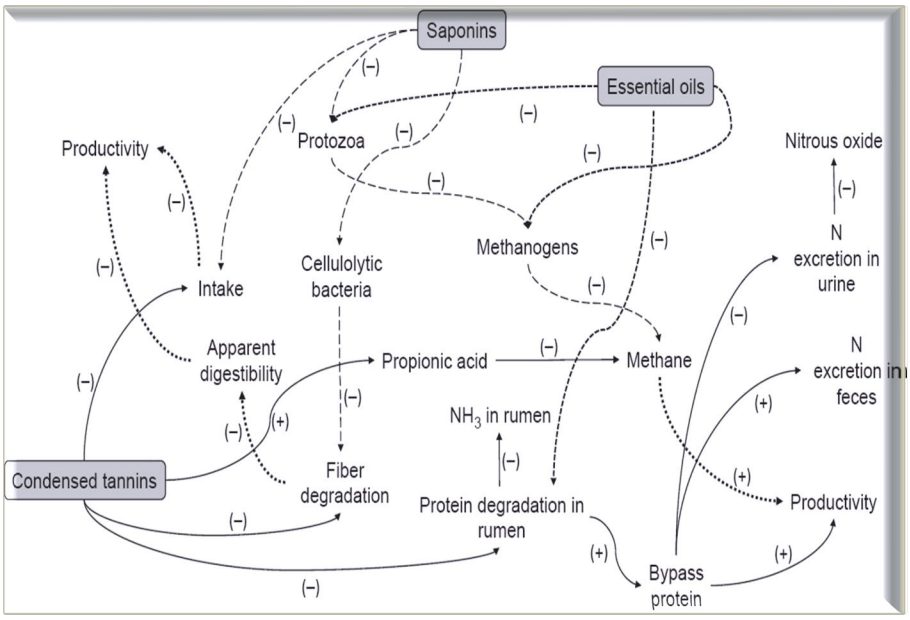

Figure 19. Metabolic and digestive effects of condensed tannins, essential oils and saponins on methane synthesis and animal production. (−): decrease; (+): increase. Adapted from (Ku-Vera et al., 2020).

Yeast addition did not significantly affect ruminal CH_4 emissions, according to meta-analysis findings of Darabighane *et al.* (2019) and Oh *et al.* (2019). In the studies conducted by Seankamsorn *et al.* (2020) and Tong *et al.* (2020), prebiotics such as chitosan, inulin, and yeast products can also reduce enteric methane emissions by altering the composition of the rumen bacterial community. While chitosan causes methanogen cell death by disrupting its cell wall permeability, yeast products, and inulin enhance the growth of other rumen bacteria that compete with methanogens for hydrogen (Vallejo-Hernández *et al.,* 2018). For the benefit of their use, more study is necessary, as their use in ruminants is still relatively insignificant in comparison to other feed additives (Sun *et al.,* 2021).

Since rumen fermentation modifiers, yeast cultures based on Saccharomyces cerevisiae have the potential to lower methane generation. Nevertheless, statistical evidence supporting the impact of yeast cultures on rumen fermentation has proven elusive in numerous instances (Vohra *et al.,* 2016). The probiotic effect of yeast cultures appears to be probably attributed to oxygen consumption by respiring yeast in the rumen (Moreno, 2012). Other approaches have recently surfaced to enhance the functioning of the rumen bacteria and regulate the digestive processes. One approach is to boost the feed's energy value while reducing cellulose content. Research has also shown that probiotic administration benefits the digestion mechanisms involved in cellulolytic digestion. Probiotic (yeast) supplementation shortens the time between

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nursing cows' meals, which speeds up the rumen's digestion of fiber and balances its acidity index (Slozhenkina *et al.,* 2022).

When high-starch diets provide it, active dry yeast, often *Saccharomyces cerevisiae*, is now widely acknowledged to have positive effects on livestock productivity, whether dairy cattle (Ferraretto *et al.,* 2012) or beef cattle (Vyas *et al.,* 2014). Through interactions with lactate-metabolizing bacteria, supplementing with ADY has been demonstrated to improve feed efficiency, rumen maturity, and ruminal pH stabilization (Thrune *et al.,* 2009). It was found that yeast can be applied to attenuate CH_4 production from dairy calves fed a high concentrate diet after in vitro trials revealed yeast addition (4 mg/g DMI decreased CH_4 production (Hernandez *et al.,* 2017). However, there was no effect of live yeast (*Saccharomyces cerevisiae*) on CH_4 emissions in beef cattle (McGinn *et al.,* 2004). Additionally, following a 24-hour incubation period, horse fecal inocula mixed with Biocell F_{53} reduced CH_4 synthesis by 78% at 4 mg/g DM but improved CH_4 production at the dose of 2 mg/g DM, according to in vitro testing (Elghandour *et al.,* 2016). Consequently, the effectiveness of ADY supplementation in lowering methane generation is still up for debate. On the other hand, since responses can differ based on yeast dosage, basal diets, and the ratio of concentrate to forage, more research is required to determine the benefits of yeast in reducing CH_4 generation in ruminants. Also mentioned at this point by Palangi *et al.* (2022).

1.5.1.4. Natural plant extract

Numerous investigations have investigated different plant parts and extracts that inhibit two processes: (1) methanogens to suppress enteric methane output and (2) ruminal protozoa and the bacteria that use AA as an energy source (Hyper-ammonia producers) to reduce protein waste (Patra *et al.,* 2017; Hartinger *et al.,* 2018). The mechanisms of action of each class of plant bioactive involve different molecules. In recent years, the cattle business has successfully substituted antibiotics and feed additives with plant and herbal extracts. The rumen microbial population and nitrogen metabolism, methane production, and overall animal health and performance have all improved with the use of plant-extracted material, including tannins (Patra *et al.,* 2012), organic sulfides (Rebelo *et al.,* 2019), essential oils (etheric oils), and saponins (Adegbeye *et al.,* 2019; Zhou *et al.,* 2020).

Abdulrahman *et al*. (2019) found that integrating eucalyptus (*Eucalyptus globulus*) oil could reduce methane output. The study investigated the impact of herbal extracts on methane production. Certain extracts and essential oils may have inhibitory effects because of their toxicity, which lowers the population of rumen microorganisms, microbial fermentation, and methanogenesis. Experimenters have peaked at the anti-methanogenic parcels of plant materials like flavonoids, tannins, essential oils, and saponins. (Manh *et al.,* 2012) documented that adding 100 g/d of eucalyptus leaf to feed for pasturing animals may be an alternate feed enhancer since it controls the appearance of rumen methane gas in cattle while maintaining mineral digestion.

The essential oils of oregano and white thyme display the possibility of replacements for ionophores in lowering methane (Benetel *et al.,* 2022; Brice *et al.,* 2022). Since they can regulate ruminal fermentation and diminish rumen methanogenesis while maintaining feed digestibility. Fraser *et al. (*2007) showed that the phenolic monoterpene found in cloves and cinnamon has antibacterial activity against Gram-positive and Gram-negative bacteria

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polyphenol, containing plant preparations decrease nitrogen levels by shielding proteins from ammonia-induced breakdown (Slozhenkina *et al.,* 2022).

Methane generation is suppressed by polyphenolic substances like flavonoids and tannins. In recent times, the generation of ammonia and methane was decreased by an iso-flavonoid-rich licorice extract; these effects had reductions in the variety and quantity of bacteria and protozoa, as well as modifications in the structure of bacteria and archaea (Ramos-Morales *et al.,* 2018). Ku-Vera *et al.* (2020) revealed that a commercial citrus extract containing flavonoids (Bioflavex®) increased propionate concentrations and the population of *Megasphaera elsdenii,* while decreasing methane production and the population of hydrogenotrophic methanogenic archaea (Suybeng *et al.,* 2019; Ku-Vera *et al.,* 2020). The inclusion of plant extracts in animal feed impacts alterations in the taxonomic makeup of the microbiome within the animal's digestive tract. It is important to remember that the rumen contains a vast diversity of microorganisms, some of which contribute positively to energy production and the formation of amino acids from non-protein forms of nitrogen, and others have increased the fermentation of the rumen and released copious amounts of methane.

1.5.2. Enzymatic inhibitors (EIs)

Halogenated methane analogs, such as 3-nitroxypropanol (3-NOP) (Pitta *et al.,* 2018), 2-bromoethanesulfonate (Lee *et al.,* 2009), and bromochloromethane (BCM) (Shima *et al.,* 2002), operate as enzyme inhibitors (EIs) by preventing the enzymatic activity required for methanogenesis and reducing methane emission.

1.5.2.1. 3-Nitrooxypropanol (3-NOP)

3-NOP, whose effectiveness is major (reducing methane emissions by up to 40%) right after feeding (i.e., maximal 3-NOP intake), decreases to a roughly 20% reduction 10 hours after feeding and is undetectable 2 hours before the meals (Hristov and Melgar, 2020). The effectiveness of anti-methanogenic supplements may also depend on the makeup of the diet. For instance, the mitigating impact of 3-NOP diminishes as diet NDF concentrations rise. It is probably acetate-type rumen fermentation that leads to more production of hydrogen. (Kebreab *et al.,* 2023). Fumarate plus 3-NOP has the potential to be a more effective method of reducing CH_4 synthesis than 3-NOP alone since it inhibits methanogenesis more and reduces H_2. However, more research is required to assess the long-term impacts of fumarate and 3-NOP on rumen fermentation, animal health, production efficiency, and the in vivo microbial community (Liu *et al.,* 2022).

A meta-analysis by Dijkstra *et al.,* (2018) on the effectiveness of 3-nitrooxypropanol in lowering methane emissions illustrated that it could decrease enteric methane by 22% in beef and 39% in dairy. 3-NOP works best against *methyl-coenzyme M reductase*, a crucial enzyme in the archaeal methanogenesis pathway (Ermler *et al.,*1997). Without changing feed intake or milk supply, its supplementation reduced daily CH_4 emissions (-39%, -29 to -47%).

In addition, 3-NOP boosted the fat in the milk of dairy cattle (Deman *et al.,* 2015) and feed efficiency in cattle raised in feed lots (Vyas *et al.,* 2018). It might potentially reduce the expense of the supplement and encourage its use.

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1.5.2.2. *Asparagopsis* and seaweeds

In freshwater and the ocean, seaweeds are multicellular, complex creatures that can reduce greenhouse gas emissions by 30% to 69%. Based on the pigments used in photosynthesis, seaweeds were classified as red algae (*Rhodophyceae*), green algae (*Chlorophyceae*), and brown algae (*Phaeophyceae*). Brown algae contain phenols, which are polyphenols or meso-phenol tannins. It has appeared that the seaweed's bioactive components lower enteric methane emissions and influence the populations of microbes in the rumen (Roque *et al.,* 2019b). However, there are other ways in which ocean seaweed cultivation can help the environment. Seaweed preserves calcifiers from acidification, eliminates excess nutrients, sequesters carbon, and offers a rich habitat for fish in the ocean (Duarte *et al.,* 2017; Park *et al.,* 2018).

Seaweed shields shorelines from wave action and offers food for many native animals (Guannel *et al.,* 2015). In a further experiment, 0.2% of organic feed matter in the form of dried asparagopsis increased weight growth by 42% and decreased methane emissions by up to 98% while having no side effects on feed intake or rumen function in cattle kept in confinement (Kinley *et al.,* 2016; Kinley *et al.,* 2020). Dairy cows fed 0.5 percent of dry matter of *Asparagopsis taxiformis* showed similar reductions in methane generation, ranging from 55% to 80% (Stefenoni *et al.,* 2021).

According to Roque *et al.*(2019a), the amount of methane produced by cows decreased dramatically with the amount of *Asparagopsis armata,* included in their milk (by 26.4% at a low level and 67.2% at a high level; the differences between the treatments were not statistically significant). As some of the fastest-growing photosynthetic creatures on Earth, seaweeds take up a lot of carbon dioxide from the water they grow in as nutrients like phosphorus, nitrogen, and heavy metals (Kim *et al.,* 2019a; Visch *et al.,* 2020). Seaweeds are estimated to absorb about 200 million tons of CO_2 annually worldwide.

It has been reported in recent years that adding seaweed to cows' diets can cut their methane emissions by 82%. Adding a tiny quantity from *Asparagopsis taxiformis* seaweeds to animal feed has been suggested. Also, the quality of the milk remained unchanged, but there was a noticeable decrease in the amount of gas produced. There are different ways of dealing with *Asparagopsis taxiformis*, which is not abundant enough in nature for industrial applications. Feed supplementing with seaweed (such as *Asparagopsis taxiformis*) is another method of inhibiting CH_4 emissions, as it can reduce daily emissions by as much as 80% (Roque *et al.,* 2021).

Seaweeds and phytoplankton in the water naturally create bromoform, the principal anti-methanogenic chemical found in *Asparagopsis* (Wever and Van der Horst, 2013). More experimenters examined the macroalgae Asparagopsis's prospect to reduce methane emissions in vivo and in vitro. With asparagopsis added at 0.5% of diet OM, pointer methane generation was lower by 26.4%, methane yield (adjusted for feed intake) by 20.5%, and methane intensity (revised for milk output) by 26.8%, all without affecting milk yield or intake. Methane production decreased by 67.2%, methane yield decreased by 42.6%, and methane intensity decreased by 60.0% when the level increased to 1%. On the other hand, milk yield and feed consumption were lowered as well.

When comparing cows that ate macroalgae to controls, there was no discernible difference in the amount of bromoform in their milk. To effectively employ *Asparagopsis* as a feed

addition, some processing needs to consider the possibility of elevated concentrations of other minerals in milk. It follows that adding seaweed to dairy cattle's diet could potentially cut their enteric methane emissions in half. There were very slight variations in carbon dioxide emissions when feed intake or milk yield made a few changes.

Studies conducted *in vitro* revealed that *Asparagopsis* also reduced the generation of volatile fatty acids (Machado *et al.,* 2016). Based on Li *et al.* (2016), as *Asparagopsis* levels rose, so did the formation of propionate, a hydrogen sink. Almost every feed supplement that lowers methane production further reduces the acetate-to-propionate ratio (Hristov *et al.,* 2015). Merely *Asparagopsis* has proven to be highly effective and significantly anti-methanogenic, even at low inclusion levels in animal diets, without adversely affecting rumen function (Kinley *et al.,* 2016; Li *et al.,* 2016).

The recommendation of seaweed cultivation as a mitigation method is pending due to the high iodine content of Asparagopsis species, which restricts its feed value in numerous countries, and the environmental consequences of seaweed cultivation (Navarro *et al.,* 2015). Due to their anti-methanogenic qualities, seaweeds referred to as macroalgae, as brown (*Phaeophyta*), red (*Rhodophyta*), and green (*Chlorophyta*) seaweeds, have gained popularity as feed additives (Vijn *et al.,* 2020; Roque *et al.,* 2021). When using *Asparagopsis taxiformis* and its fellow *Asparagopsis spp*., which could reduce in vivo methane emissions in dairy cattle from 50% to over 80% (Maia *et al.,* 2016; Abbott *et al.,* 2020; Min *et al.,* 2021), several in vitro studies of seaweed supplements showed a negative correlation with methane generation (Kinley *et al.,* 2020).

1.5.3. Hydrogen receptors (HRs)

HRs include nitrates, organic acids such as fumaric acid (FA), and unsaturated lipids (Castillo *et al.,* 2004).

1.5.3.1 Nitrate

This meta-analysis's findings show that nitrate supplementation in dairy and beef cattle decreased CH_4 emissions in a dose-dependent manner, both in terms of output in g/d and yield in g/kg of DMI. So, it concerned CH_4 generation and yield, nitrate had a more moderating influence on dairy cattle than beef cattle. Indeed, slow-release nitrate in beef cattle appears to be attached to the type of cow. The higher nitrate dose increases the mitigating effect of nitrate on CH_4 generation and yield, while a higher DMI decreases this effect (Feng *et al.,* 2020).

Li *et al.* (2013) and Feng *et al. (*2020) demonstrated that nitrate can successfully reduce CH_4 over an extended period and reduce daily CH_4 emissions in a dose-responsive manner without compromising its effectiveness. Nitrate is more successful than 3-NOP in reducing daily CH_4 emissions in dairy cattle than in beef cattle (Ungerfeld, 2020). So, if animals are not properly adapted, toxicity might occur. Early studies on nitrate supplementation in ruminant diets indicated a drop in feed intake and no toxicity signs, even though nitrate can be harmful (Van Zijderveld *et al.,* 2011). Dietary nitrate supplementation can also reduce the amount of crude protein in the diet, eventually lower methane emissions, and increase productivity (Hadipour *et al.,* 2021).

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1.5.3.2 Organic acids: malate and fumarate

Biological tissues frequently contain malate and fumarate, two salts of the four-carbon dicarboxylic acids that act as intermediaries in the citric acid cycle (Crespo *et al.,* 2002). Some anaerobic bacteria synthesize succinate or propionate as a source of biosynthetic precursors via a reductive or reverse citric acid cycle known as the succinate–propionate pathway (Nelson and Cox, 2000). Important intermediates in this metabolic process that *S. ruminantium* employs are fumarate and malate.

These organisms use the first three citric acid cycle processes to produce ketoglutarate; because they are deficient in ketoglutarate dehydrogenase, they have no chance to complete the citric acid cycle reactions. However, by reversing the "normal" (oxidative) direction of flow through the cycle, they can make malate, fumarate, succinate, and succinyl-CoA from oxaloacetate since they have the four enzymes needed to catalyze the reversible conversion of oxaloacetate to succinyl-CoA (Nelson and Cox, 2000). As propionate precursors, organic acids like fumaric and malic acid compete with hydrogen to reduce CH_4 emissions. Raising the malic acid content reduced the amount of CH_4 produced in the rumen when various diets were allowed to ferment (Wu *et al.,* 2015).

Fumaric acid is more effective than malic acid at reducing the generation of CH_4 by as much as 42% (Foley *et al.,* 2009). However, compared to in vivo investigations, the reported effectiveness of organic acids in vitro is lower, possibly primarily a product of a lower reaction rate for hydrogen utilization than for hydrogen synthesis (Sun *et al.,* 2023). The rumen metabolism converts fumarate, an intermediary, to propionate in the final stage (Yang *et al.,* 2012). Compared to the methanogenesis of H_2 and CO_2, fumarate reduction yields more Gibbs-free energy and has a lower H_2-consuming threshold (0.02 ppm). In the rumen, fumarate reduction ought to be more efficient than methanogenesis (Ungerfeld and Kohn, 2006). Fumarate has been used as a rumen CH_4 mitigation agent (Molano *et al.,* 2008). Fumarate's effects on rumen CH_4 mitigation, however, were discovered to be variable (Bayaru *et al.,* 2001). Rumen fermentation is boosted by applying organic acids, which activate the rumen microorganisms.

A fascinating topic that merits further research is whether using fumaric acid and malic acid has a positive effect on rumen feed conversion parameters (Akyurek and Salman, 2021). Because organic acids are good for animal growth and health, they were placed as an alternative feed supplement in the diets of ruminants. Examples of these acids are fumaric acid and malic acid. In the rumen, fumarate is first reduced to succinate and then undergoes decarboxylation to become propionate, using hydrogen in the process ($C_4H_4O_4 + 2H \rightarrow C_3H_6O_2 + CO_2$) (Yin *et al.,* 2015).

Methanogens will make less [H] available to them when restored fumarate. Fumarate can suppress methanogenesis by shifting [H] from methanogenesis to propionate synthesis as an alternate H_2 sink (Castillo *et al.,* 2004).

Naturally occurring substances, FA and MA, are involved in the metabolism of plant and animal cells. According to Castillo *et al.* (2004), these acids substitute for the use of antibiotics by changing the reproductive microbial ecology and improving the well-being and production of animals. Natural foods for plant and animal tissues include organic acids.

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Their benefits in improving feed quality and animal performance, decreasing ruminal methane emissions, and altering the microbiota balance and numbers in the gastrointestinal tract have led to their usage as a stimulant in animal diets (Martin, 1998). In the work of Castillo *et al.* (2014), FA and MA have ruminal anti-methanogenic actions because they function as hydrogen acceptors, propionate precursors, and intermediates in the pathway from succinate to propionate. Malic acid was added to the aqueous material during the silage of the maize product up to 1.5%, which reduced the amount of methane produced *in vitro* while having little impact on feed digestion or total gas generation in vitro (Kara *et al.,* 2015). MA alone may be a more effective anti-methanogenic feed additive in lowering methane emissions, while fumaric acid and malic acid together also reduce methane production by inhibiting feed digestion, according to study data. Lower fumaric acid dosages may have an anti-methanogenic impact (Kara *et al.,* 2022).

Numerous *in vitro* investigations have demonstrated that malic acid can raise feed digestibility, enhance microbial N (Sniffen *et al.,* 2006), and raise rumen pH (Carro and Ranilla, 2003). Fumarate supplementation resulted in higher amounts of acetic acid and ruminal propionate in addition to a decrease in methane production (Newbold *et al.,* 2005). Previous research (Carro and Ranilla, 2003) has demonstrated that citric and malic acids can promote rumen fermentation and improve feed digestibility. Wang *et al.* (2019a, b) supported the beneficial benefits of the MA and CA supplements on milk production. It demonstrated that applying MA and CA during ensiling gives animals access to extra feed additives that may improve animal performance (Ke *et al.,* 2017).

Furthermore, fumarate has the potential to lower methane production and broaden the variety of bacteria in the rumen (Mao *et al.,* 2008). *Fumarate reductase* may convert fumarate to succinate as an intermediary in the propionate pathway, and certain microbes may then decarboxylate to propionate (Mao *et al.,* 2008). Fumaric acid may act as an intermediary during the fermentation of silage, according to research that found fumaric acid in ensiled forages (Guo *et al.,* 2020). Farm animal feeds contain organic acids for a variety of uses. In contrast to silage, organic acids raise the diet's acidity and stop it from degrading. Formic acid proved to lower the overall amount of gas produced in vitro at low dosages but to have the opposite effect at greater concentrations (Kara *et al.,* 2015). Malate and fumarate have two crucial roles: they supply the carbon required for cell synthesis processes and the electron sink needed to produce succinate.

Fumaric acid has the potential to be used sustainably by lowering ruminant methane emissions and enhancing environmental conditions (Palangi and Macit, 2021). Propionate precursors, sometimes known as "propionate enhancers," are intermediates in the fermentation pathways leading to propionate and have been studied as potential means to modify hydrogen in the rumen (Garcia-Martinez *et al.,* 2005; Newbold *et al.,* 2005). As fumarate-reducing bacteria, *Veillonella parvula, Fibrobacter succinogenes*, and *Selenomonas ruminantium subsp. lactilytica* has demonstrated a significant capacity to reduce fumarate and should boost propionate formation in the rumen.

Furthermore, intermediates that help turn pyruvate into butyrate (also known as "butyrate enhancers") may use hydrogen as an electron sink (Carro and Ungerfeld, 2015). It is necessary to assess CH_4 mitigation techniques from several angles regarding application scenarios, potentials, and dosages or concentrations of utilization. Dietary approaches can cause ruminal acidity, change the pH of the rumen, and reduce the palatability of the meal (Sun *et al.,* 2023).

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Upon inhibition of methanogenesis, the primary alternative metabolic pathway in the rumen for distributing excess H_2 are reductive acetogenesis, propionogenesis, reduction of nitrate and sulfate, formate accumulation, and an increase in microbial biomass production (Newbold *et al.,* 2005; Leng, 2014; Gagen *et al.,* 2015; Ungerfeld, 2015).

1.5.3.3 Oils

Numerous studies have examined essential oils, and they may have some use in dairy nutrition. Combinations of plant bio-actives may increase consistency and efficacy while reducing the overall inhibition caused by each ingredient (Beauchemin *et al.,* 2020). In vitro studies show that essential oils derived from thyme, garlic, eucalyptus, oregano, or cinnamon can reduce methanogenesis. However, very few have been shown to possess persistent anti-methanogenic properties in vivo (Cobellis *et al.,* 2016). Plant components such as flowers, seeds, buds, leaves, herbs, timber, fruits, twigs, and roots can yield volatile and aromatic oily liquids called essential oils (EOs) (Burt, 2004). The broad-spectrum antibacterial qualities of EOs make them safe for ingestion by humans and animals (Davoodi *et al.,* 2019).

Certain microbes, including methanogens, respond to EOs in distinct ways by encouraging or suppressing several groups of microorganisms (Benchaar and Greathead, 2011). Some indirectly prevent protozoa from growing, or they do so by bio-hydrogenating unsaturated fatty acids, which reduces the amount of hydrogen available to methanogens (Toprak, 2015). Essential oils improve dairy cow performance and lower methane emissions (Hart *et al.,* 2019). A considerable drop in methane and CO_2 production occurred with increasing amounts of essential oils given to the feed in a study examining the effects of thyme (*Thymus vulgaris*), mint (*Mentha piperita*), and orange (*Citrus sinensis*) oils on rumen fermentation (Canbolat *et al.,* 2011).

The main benefits of essential oil for ruminants are that it raises propionate and lowers CH_4, acetate, and NH3-N without impairing volatile fatty acids (Eburu, Patrick Okara, 2016). The concentrations, types, dosages, and species of microbes in the ruminal fluid determines how well an essential oil inhibits the growth of rumen bacteria. Citrus essential oils have limited rumen fermentation, lower rumen ammonia, and volatile fatty acids, and have no negative impact on body weight, dry matter intake, or the overall digestibility of nutrients in Hu sheep (Wu *et al.,* 2018).

According to Busquet *et al*. (2005a), after two hours of feeding, 2.2 mg/L of carvacrol dramatically decreased peptide-N and raised NH3-N, suggesting either an inhibition of proteolysis or a promotion of peptide lyses. It was recognized that eugenol, anethol, and cinnamon alkalize have antibacterial properties against gram-positive and gram-negative microorganisms. The active component of cinnamon oil, cinnamonldehyde, can alter the rumen microflora's N-metabolism by inhibiting peptide lysis, so it has no detrimental effects on the proportions of total volatile fatty acids (Cardozo *et al.,* 2004). Greater antibacterial efficacy at higher cinnamaldehyde dosages was associated with lower level of total volatile organic compounds (Busquet *et al.,* 2003).

There is proof indicating the effects of cinnamon oil and cinnamonaldehyde on the relative concentrations of each volatile fatty acid, propionate, butyrate, and acetate. Cinnamaldehyde increases propionate with no effect on acetate or butyrate proportions, whereas cinnamon oil has an increasing impact on acetate with no effect on propionate or butyrate proportions (Calsamiglia *et al.,* 2007). At low concentrations, cinnamon aldehyde demonstrated a non-

significant difference in the proportion of each VFA over an extended period (Busquet *et al.*, 2005a, b). However, at higher concentrations of 31.2 and 312 mg/L, cinnamon aldehyde significantly decreased the proportion of acetate and increased the proportions of propionate and butyrate (Busquet *et al.,* 2005b).

A few studies have shown that low amounts of lipid supplementation (4% of dietary dry matter intake) might improve the energy density of diets and, in some cases, improve animal productivity while reducing methane emissions by up to 20% (Grainger and Beauchemin, 2011; Patra, 2013). Increasing ruminal CH_4 emissions and milk saturated fats with plant oils in grass silage increase the proportion of unsaturated fats and conjugated linoleic acid without changing digestibility, rumen fermentation, the number of rumens microbials, or milk production (Bayat *et al.,* 2018).

Researchers discovered that adding 5% rapeseed oil (RSO) to the meals of dairy cows could cut CH_4 emissions by up to 23%. Furthermore, meals containing RSO decreased methane emissions (from breath and faeces). The previously mentioned oil addition resulted in a decrease in overall dry matter and nutrient consumption. Another study discovered that supplementing with rapeseed oil decreased the intake of dry matter and nutrients, the energy-corrected milk yield, the composition and yield of milk fat and protein, and the general digestibility of nutrients, except crude protein (Ramin *et al.,* 2021). Oil in the meal increased the relative number of *Methanosphaera* and *Succinivibrionaceae* in the rumen and decreased the abundance of *Bifidobacteriaceae*. It also reduced the daily emission and intensity of methane. Feeding the dairy cows 41 g of rapeseed oil per kg of dry matter as a dietary supplement decreased their daily CH_4 emissions by 22.5% (Chagas *et al.,* 2021).

In their *in vitro* investigation, Poulsen *et al.* (2013) raised the relative abundance of *Methanosphaera* and *Methanobrevibacter* and decreased methane in correlation with a decline in *Thermoplasmata Methanomassiliicoccaceae*. Methane-producing *Methanosphaera* and *Methanobrevibacter* increased, according to studies using lipid insertion to forage (Difford *et al.,* 2016; Bowen *et al.,* 2020). Adopting a diet high in fat and oil reduced daily CH_4 emissions. Despite a decrease in feed intake and fiber digestibility, there was no effect on weight gain in developing animals or milk output in dairy animals.

Only including primarily vegetable oils in the diet to minimize daily CH_4 emissions among the subcategories listed here. Increased availability of highly digestible, non-fermentable energy, reduced feed intake and fiber digestibility, and suppression of methanogenesis by unsaturated (or medium- chain saturated) fatty acids, which are often plentiful in vegetable oils, are all responsible for this effect. According to reports, oil inclusion lowers daily CH_4 emissions over time (Liu *et al.,* 2011) and in a dose-response manner (Eugène *et al.,* 2008).

Nevertheless, there is a limit to the amount of oil fed to ruminants, and the productivity and health of the animals are necessary to maintain a good rumen fermentation process. The highest levels of oil in ruminant diets are influenced by the physiological stage of the animal (Palmquist and Jenkins, 2017).

The four vegetable oils (sunflower oil, canola oil, linseed oil, and coconut oil) effectively reduced daily CH_4 emissions. Because the cost of oil, meat, and milk varies by location and country, the cost-effectiveness of feeding oils to reduce daily CH_4 emissions varies. Adding oils to the diet to reduce enteric CH_4 emissions was not cost-effective; however, from the standpoint of human health, trade-offs caused by concurrent improvements in the fatty acid

profile of milk and meat might help to support the use of certain oils and oilseeds (Wang *et al.,* 2014). The combination of essential oils raised the concentration of milk fat, leading to a decrease in the intensity of enteric CH_4 emission per unit of milk fat (Silvestre *et al.,* 2023).

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2. CLIMATE CRISIS

2.1. CONCERN ABOUT CLIMATE CHANGE

The world is facing a climate emergency. Furthermore, climatic hazards such as temperature variations, erratic precipitation, altered seasonal patterns, altered disease distribution, desertification, oceanic effects, and soil and coastal area degradation make many countries more vulnerable in a variety of sectors, according to the United Nations Climate Change Secretariat (UNCCS, 2019). Given the fluctuations between years, seasons, and months, it has been stated that it is impossible to directly feel the long-term trend of climate change since it is a long-term process that occurs over a significant amount of time (Hansen *et al.,* 2012). Without a doubt, climate change is today's most pressing concern. As expected, the World Economic Forum regards climate inaction as a greater threat to humanity than the use of weapons of mass destruction. In 1988, scientist James Hansen of the National Aeronautics and Space Administration (NASA) highlighted the 'greenhouse effect,' or rising Earth's temperature. (see Washington Post, August 3, 2012; **Image 3**)

Image 3. In 1989, James Hansen, a global warming expert, delivered a speech to the US Congress

The scientific challenge of the climate calamity is intricate. It is, and it varies according to daily or seasonal weather conditions. The measurement of greenhouse gases (GHG) and the United Nations Framework Convention on Climate Change (UNFCCC, 2015) have provided scientific proof that "human-induced climate change" is taking place (IPCC, 2014b; Satgar, 2018). In a practical study, the Intergovernmental Panel on Climate Change (IPCC) assessed the risks and consequences of various levels of global warming: 1.5 °C-2 °C (IPCC, 2018).

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2.2. HISTORY OF CLIMATE CHANGE CONCERN

In just 150 years, the pre-industrial CO2 level of the atmosphere increased from 280 parts per million to 420 parts per million. Since the industrial revolution, atmospheric CO_2 concentrations have increased by 47%, while methane (CH_4) and nitrous oxide (N_2O) concentrations have increased by 23% and 156%, respectively **(**IPCC, 2021; Bilotto *et al.,* 2023). "Nature is the most important source of mathematical discoveries," says Jean-Baptiste Joseph Fourier (1768-1830, **Image** 4) (Sitaram, 1998).

Image 4. Fourier was the first to provide a scientific explanation for the greenhouse effect.

In publications published in 1824 and 1827, Fourier investigated numerous probable origins of the reported leftovers of heat and proposed that interstellar radiation could account for much of the additional heat. Fourier considered the potential of the environment to function as an insulator, which is regarded as the first description of the greenhouse effect.

Another scientist and women's rights campaigner, (**Image 5**) Eunice Newton Foote (1819-1888), took thirty years to become interested in the hypothesis that the atmosphere might hold heat. His small study is titled Circumstances Affecting the Heat of the Sun's Rays.

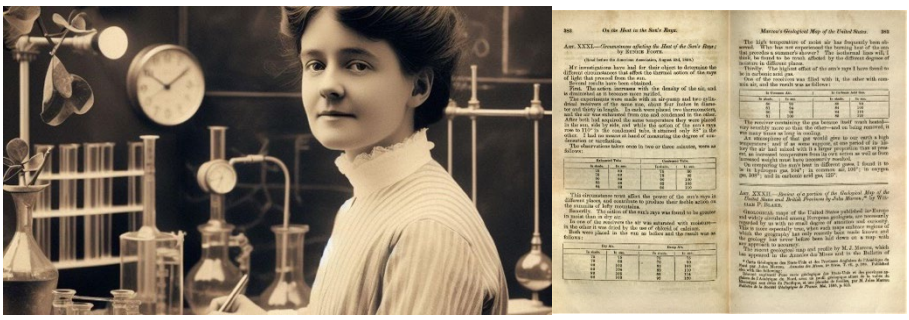

Image 5. Eunice Foote anticipated by three years the tests of the Irish-born British physicist and chemist John Tyndall, who is now regarded as the discoverer of the effect of CO_2 in the atmosphere and on which the Irishman most likely relied three years later.

After three years, Irish physicist John Tyndall (1820–1893) became active in climate science. Tyndall (**Image 6**) is one of those few scientists whose names appear in so many threads that you wonder if they are the same fellow. Tyndall discovered that coal, a combination of methane and other gases, absorbs infrared radiation. Tyndall felt that the ice ages caused

atmospheric changes, consistent with scientific concerns at the time. He was concerned about the consequences of even minor increases in carbon dioxide levels (Jackson, 2020). In 1859, John Tyndall discovered that gases like carbon dioxide, methane, and water vapor block infrared light, whereas oxygen and nitrogen do not. Tyndall is widely credited with developing the technique for absorbing greenhouse gases in the atmosphere.

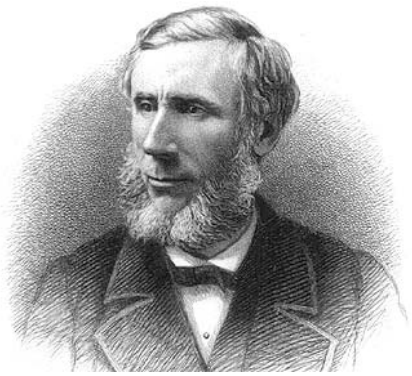

Image 6. Tyndall is commonly regarded as the discoverer of the mechanism for the absorption of greenhouse in the atmosphere.

Image 7: Many experts believe Arrhenius' experiments were ahead of their time. His research into the agents responsible for global warming, as well as his recommendations for averting this significant issue that is currently harming life on Earth, are examples of this.

Svante Arrhenius (**Image 7**), a Swedish mathematician who lived from 1859 to 1927 and was the first Swede to win the Nobel Prize in 1903, eventually analyzed the findings of Fournier and Tyndall. In 1895, Arrhenius attempted to assess this impact on the greenhouse effect, which involved doing numerous and time-consuming calculations by hand. He determined that if atmospheric CO_2 levels were reduced to half of what they were back then, the planet's temperature would drop by 4 to 5 degrees Celsius, perhaps triggering a major cooling like what happened during glaciation.

Scientists' views were leaning more in favor of the warming theory. By the 1990s, most scientists agreed that the greenhouse effect played an important role in most climate changes and that human emissions posed a significant risk to global warming, thanks to improved computer model fidelity and observational evidence supporting Milankovitch's theory (**Image 8)** of glacial epochs. Since then, most of the scientific research has been focused on the release of the Intergovernmental Panel on Climate Change reports (Paz, 2023).

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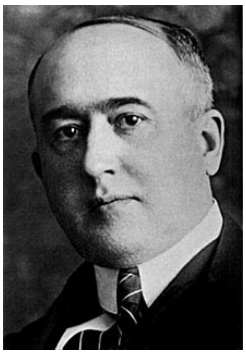

Image 8: Milutin Milanković was well known for his theory of the Ice Ages, which links variations in the Earth's orbit and long-term climate changes.

2. 3. ENVIRONMENTAL CHANGES

Rising temperatures and dwindling biodiversity, collectively known as "nature loss," pose significant threats to human life. In keeping with the planetary health concept, we propose a cross-disciplinary approach to preventing health and environmental disasters (Whitmee *et al.,* 2015). However, there is a recognition of the existential concerns posed by environmental loss and climate change (IPCC, 2014a; Shin *et al.,* 2019). A few practical steps can lead to a sustainable and healthy ecosystem. Climate change-related environmental challenges have made it more difficult for humans to exist and achieve sustainable development. Climate change is a global concern due to the recurring occurrence of climatic events such as droughts, floods, and irregular precipitation patterns, as well as the expectation of disruptions to crop growth, phenology, and irrigation systems (Babel *et al.,* 2019; González- Hernández *et al.,* 2022; Wang *et al.,* 2022;). Climate change will most likely affect the agricultural system (Ahmadi *et al.,* 2022; Ho *et al.,* 2022). The IPCC. (2018; 2023) states that irrefutable proof that global warming is attributed to human activity (**Figure 20**). The Earth's mean surface temperature is estimated to have increased by 1.2°C since pre-industrial times, rising more on land than in the ocean. Depending on the trajectory of global greenhouse gas emissions, it is predicted to consistently approach 1.5°C during the 2030s (IPCC, 2023).

The year 2023 surpassed all previous periods through temperature measurements as the warmest year ever, with temperatures nearly 1.5°C above pre-industrial levels. 2024 might even be warmer than 2023 due to the warming effects of the present El Niño weather trend in the equatorial Pacific. Consequently, the primary greenhouse gases in the atmosphere such as carbon dioxide, nitrous oxide, and methane continue to increase in concentration. As an example, according to data compiled by the US National Center for Environmental Information (NCEI), in February 2024, the global land surface temperature for January was 1. 27°C above the 20th-century average, which puts it above the January 2016 record. According to NCEI's Annual Global Temperature Outlook, there is a 22% chance that 2024 will be the warmest year on record and a 99% chance that it will be in the top five.

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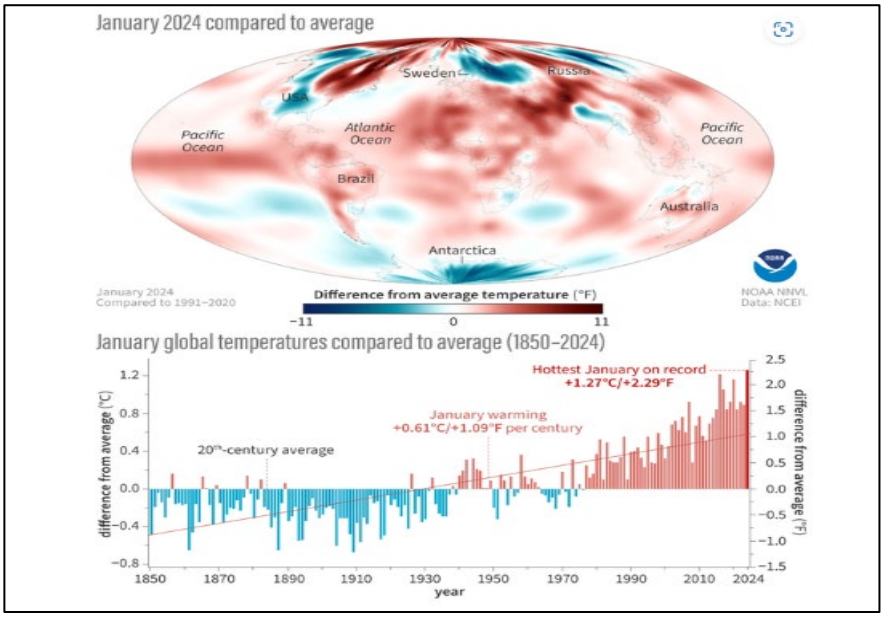

Figure 20. Depicts global surface temperatures for January 2024 compared to the 1991-2020 average, with a few colder-than-normal areas (blue) dispersed among largely warmer-than-average temperatures. The average January temperature increased by 0.61 ˚C per century. Source by (https://www.climate.gov/news-features/understanding-climate/global-climate-summary-january-2024.

Meanwhile, air pollution from industry, transportation, and energy production has progressively decreased due to worldwide regulatory initiatives in recent decades. The International Maritime Organization (IMO) made a significant decision to implement new regulations that started in January 2020 (Hansen *et al.,* 2023a; 2023b).

Global warming has already displaced millions of people worldwide, and this displacement will only grow in breadth and size, resulting in ever-larger migrations within and between states. Several weather disasters in recent years have harmed millions of people, exacerbated by climate change. Furthermore, climate change increases and magnifies dangers by exacerbating existing problems. It increases material hardship, instability, vulnerability, and unfairness, for example, while exacerbating pre-existing hazards like fire and flooding. Critically, climate change has all the hallmarks of a "long problem" (Hale, forthcoming), a "creeping problem" (Boston, 2023), and a "super-wicked problem" (Lazarus, 2008). Firstly, its essence is genuinely multigenerational.

While it is possible to achieve geological net zero emissions globally this century and even in the next four to five decades, the issue of adaptation will persist for centuries, if not millennia, in one form or another.

In the same way, climate change is a slow-burning issue or a disaster with a gradual commencement. Rather, we could be dealing with a protracted sequence of negative occurrences. It is preferable to cut global greenhouse gas emissions as soon as possible and to adopt techniques of carbon capture and storage (Gambhir *et al.,* 2023; IPCC, 2023). However, even with swift and continuous decarbonization, people will adjust to increasingly severe climate change-related occurrences and learn to live with them. Specifically, the international world needs to get ready for the massive destruction of coastal homes and infrastructure, as well as the possibility of hundreds of millions of people having to move.

Climate change is associated with the acceleration of environmental changes, the rise in atmospheric carbon dioxide (CO_2) concentration and its anomalous warming, the greenhouse effect, and the expansion of decertified regions. Different studies show that, with a significant anthropogenetic influence, climate change-related variations in rainfall and temperature exacerbate worldwide trends. The world has already experienced a global warming of 1.0°C above pre-industrial levels, with variations between 0.8 and 1.2°C, according to reports from the United Nations (UN), the Intergovernmental Panel on Climate Change (IPCC), and the World Meteorological Organization (WMO) (Rodrigues do Nascimento, 2023). The dangers to constructed infrastructure, livelihoods, critical services, and the safety of food via water are increased by climate change. Global average temperatures are rising as the frequency, severity, and length of heat waves are rising due to climate change (Perkins-Kirkpatrick and Lewis, 2020).

Worldwide warming and the observed and predicted climate changes for the twenty-first century are two of the most significant global developments during the previous 65 years. With its impact on several aspects of the ecological, environmental, socio-political, and socio-economic disciplines, climate change is an international intergovernmental complex challenge (Adger *et al.,* 2005; Leal Filho *et al.,* 2021; Feliciano *et al.,* 2022).

2.4. SOURCES OF GREENHOUSE EMISSIONS

The Environmental Protection Agency (EPA, 2017) defines human health risk assessment as the procedure of estimating the nature and probability of disadvantageous health belongings in humans who may be exposed to chemicals in polluted environmental media, presently or in the future (US EPA, 2021; LaKind *et al.,* 2023). Greenhouse gases are referred to gases that trap heat in the atmosphere (US EPA, 2021). The atmosphere contains synthetic gases (**Table 2**) like perfluorocarbons (PFCs), hydrofluorocarbons (HFCs), and chlorofluorocarbons (CFCs), as well as sulfur hexafluoride (SF6).

The release of GHGs into the atmosphere, such as CO_2, CH4, N_2O, and H_2O, was thought to have originated from natural sources such as volcanoes, forest fires, and seismic activity before the industrial revolution (Murshed *et al.,* 2020; Sovacool *et al.,* 2021; Murshed, 2022; Usman and Balsalobre-Lorente, 2022). Greenhouse gases are a key cause of climate change (Li *et al.,* 2010; Alemu *et al.,* 2011; Okpara, 2018; NOAA, 2020). In contrast to other gases like CH_4 and nitrous oxide, which stay in the atmosphere for over a century, CO_2 stays in the atmosphere for 40% of a century and 20% for a millennium after emission by the Global Warming Potential (GWP, 2022).

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Table 2: Chemically reactive greenhouse gases and precursors: abundances, trends, budgets, lives, and 100-year horizon values (GWP). Source by (https://www.ipcc.ch/site/assets/uploads/2018/03/TAR-04.pdf).

Chemical species	Formula	Abundance[a] ppt 1998	Abundance[a] ppt 1750	Trend ppt/yr[a] 1990s	Annual emission late 90s	Life-time (yr)	100-yr GWP[b]
Methane	CH_4(ppb)	1745	700	7.0	600 Tg	8.4/12[c]	23
Nitrous oxide	N_2O (ppb)	314	270	0.8	16.4 TgN	120/114[c]	296
Perfluoromethane	CF_4	80	40	1.0	~15 Gg	>50000	5700
Perfluoroethane	C_2F_6	3.0	0	0.08	~2 Gg	10000	11900
Sulphur hexafluoride	SF_6	4.2	0	0.24	~6 Gg	3200	22200
HFC-23	CHF_3	14	0	0.55	~7 Gg	260	12000
HFC-134a	CF_3CH_2F	7.5	0	2.0	~25 Gg	13.8	1300
HFC-152a	CH_3CHF_2	0.5	0	0.1	~4 Gg	1.40	120
Important greenhouse halocarbons under Montreal Protocol and its Amendments							
CFC-11	$CFCl_3$	268	0	−1.4		45	4600
CFC-12	CF_2Cl_2	533	0	4.4		100	10600
CFC-13	CF_3Cl	4	0	0.1		640	14000
CFC-113	$CF_2ClCFCl_2$	84	0	0.0		85	6000
CFC-114	CF_2ClCF_2Cl	15	0	<0.5		300	9800
CFC-115	CF_3CF_2Cl	7	0	0.4		1700	7200
Carbon tetrachloride	CCl_4	102	0	−1.0		35	1800
Methyl chloroform	CH_3CCl_3	69	0	−14		4.8	140
HCFC-22	CHF_2Cl	132	0	5		11.9	1700
HCFC-141b	CH_3CFCl_2	10	0	2		9.3	700
HCFC-142b	CH_3CF_2Cl	11	0	1		19	2400
Halon-1211	CF_2ClBr	3.8	0	0.2		11	1300
Halon-1301	CF_3Br	2.5	0	0.1		65	6900
Halon-2402	CF_2BrCF_2Br	0.45	0	~ 0		<20	

Agriculture systems contribute significantly to GHG emissions into the atmosphere, making up around 30% of all anthropogenic emissions (including indirect emissions related to changes in land use) (Rosenstock *et al.,* 2016). Aerenchyma tissue, which serves as conduits for CH_4 and aids in oxygen uptake, is present in the roots of most wetland plants (Korrensalo *et al.,* 2022). In both natural and regenerated vegetation, vascular plants have a role in the generation and uptake of CH_4 (Wilson *et al.,* 2016; Zhang *et al.,* 2022a, b).

Wetlands are important ecosystems that preserve biodiversity in the environment and control hydrogeographic basins (Taillardat *et al.,* 2020). For most organisms, soil serves as their global home, and the biogeo cycle is dependent on the locality of the plants and soil (France *et al.,* 2022). Through topological conditions, these habitats maintain the diversity of endemic species and contribute to CH_4 emissions (Ribeiro *et al.,* 2020; Singh *et al.,* 2018a, b).

Because of variations in greenhouse gas efflux because of shifts in species and vegetation, wetlands constitute sources of CH_4 emissions (Baker-Blocker *et al.,* 1977). Humic acid builds up in wetlands due to the deep-seated anoxic conditions and facilitates carbon sequestration. In non-vegetated areas, the stored carbon outweighs the generation of CH_4 (Laanbroek, 2010). When temperatures rise, changes in organic matter impact wetlands' vegetative structure, which increases CH_4 emissions (Kandel *et al.,* 2019). Another source of greenhouse gas emissions is the determination of the redox and anoxic/toxic limits by the water table. In comparison to deeper groundwater, shallow water levels release more CH_4 and a large amount of N_2O (Prananto *et al.,* 2020).

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Methane's year atmospheric lifespan is brief (Myhre *et al*., 2014a). By using tropical hydroxyl radicals (OH) for oxidation, a process known as hydroxyl oxidation, 80–89% of the total amount of CH_4 in the atmosphere is eliminated (Kirschke *et al.,* 2013; He *et al.,* 2020).

Based on Wu *et al.* (2020) report that additional sinks comprise soil uptake, interactions with chlorine atoms in the marine boundary layer, and reactions with oxygen and chlorine atoms in the stratosphere. Although methane is a clean, productive biochemical and biofuel resource, fossil fuels deplete more quickly and backup supplies have been discredited by science (Fan *et al.,* 2021; Liu *et al.,* 2021).

Lee and Holder. (2001) have shown that CH_4 is a very efficient fuel for burning, releasing the least amount of CO_2 per mole when burned. Additionally, methanogenic bacteria offer a platform for energy conversion, which may develop into a CH4-based biomanufacturing sector in the future (Nguyen and Lee, 2021). Without utilizing the environment, CH_4 generates methanol, syngas, ammonia, and hydrogen (Richard *et al.,* 2021). Future single-cell protein manufacturing could primarily use CH_4, which revitalises rural populations and provides easy access to it (Pieja *et al.,* 2017; Kulkarni and Ghanegaonkar, 2019).

We are about to witness a profound technological shift that is required to save the environment and human beings (Jones *et al.,* 2022). Dams are constructed to store water for irrigation and to meet human needs. Building reservoirs is a result of unchecked population growth, which may result in higher water use (Jarveoja *et al.,* 2016). Aside from generating power, dams also support a variety of other uses, including fish farming, fire safety, preventing erosion, storing mine tails, and flood control. The buildup of organic matter in dams and the expulsion of CH_4 are directly related. The organic stuff that has been deposited traps greenhouse gas emissions (Fearnside and Pueyo, 2012).

Sediment accumulates at the bottom of the dam's water column because there is little agitation and airflow (Maeck *et al.,* 2013). As a result, it acts as a hot point for biological processes involved in anaerobic breakdown. Consequently, during the dam's lifetime, CH_4 is discharged straight into the atmosphere and does not diminish (Chen *et al.,* 2011). Even though its primary function is to provide green energy, it also releases a substantial amount of greenhouse gases into the atmosphere, which has an unfavorable impact on it (Rooney-Varga *et al.,* 2018). A fermentation produces CH_4 in ruminants at a similar rate of 95% and in non-ruminants at 5% (Zhao and Zhao, 2022). Anaerobic archaeal methanogens use a simple oxidation pathway to convert the single carbon source to CH_4. To break down their diet, ruminants use three different processes: (i) converting CO_2 into H_2; (ii) transforming fatty acid chains like acetic, formic, and butyric acid; and (iii) synthesizing molecules like methanol and ethanol (Lan and Yang, 2019). In the rumen, most methanogens engage (a main pathway) to convert CO_2 to CH_4.

In addition to contributing to Earth's atmosphere's global warming, nitrous oxide (N_2O) also plays a part in the formation of tropospheric ozone, which has a detrimental impact on the environment and human health (Li *et al.,* 2017). Furthermore, N_2O stability in the troposphere aids in the stratosphere's ozone layer depletion, resulting in dermatological issues related to UV exposure (Young, 2009).

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2.5. CHANGES IN TEMPERATURE RECORDS OVER THE LAST 5 YEARS

With the growth rates that occurred between 1945 and 1990, CH_4 atmospheric concentration has increased significantly since 1800 (Etheridge *et al.,* 1998). It reveals that if emissions match the amount of airborne matter that is oxidized and eliminated, there will be no net warming.

According to Cain *et al.* (2019), a CH_4 emission source will not cause warming in 20 years if it has an annual drop rate of 0.3%. General climate models predicting increased global temperatures towards the end of the twenty-first century also show signs of potential disruptions to the hydrological cycle. Methane released from agriculture accounts for 31% of global methane emissions, and 78% of this comes from enteric fermentation, primarily from the rumen of cows, the suppression of methane production is one of the main goals in the cattle industry for reducing greenhouse gases from the agriculture sector (UNFCCC, 2015).

With a 28-fold higher global warming potential than CO_2 over a 100-year horizon (GWP100), methane effectively traps radiation (Pörtner *et al.,* 2022). Raising cattle produces about 80 million metric tons of CH_4 annually worldwide, which makes up about 33 percent of the CH_4 emissions caused by human activity (Beauchemin *et al.,* 2009). The agriculture sector produces 55% of the total amount of CH_4. Global agriculture is the industry most responsible for 30–40% of greenhouse gas emissions, making it both a contributor to and a victim of climate change (Ortiz *et al.,* 2021). Extreme precipitation events, such as floods, forest fires, and droughts, have influenced several agro-environmental and climatic elements that are prevalent in agriculture productivity (Huang, 2004; Pautasso *et al.,* 2012).

Furthermore, the extreme reliance on finite resources feeds the fire and makes the world's agriculture vulnerable to destruction. The IPCC assessment states that throughout the past few centuries, atmospheric concentrations of greenhouse gases CH_4, CO_2, and N_2O have increased to unprecedented levels in the air (Usman and Makhdum, 2021).

2.5.1. Globally

Livestock growth rates, reproductive and productive efficiency, illness and mortality, and feed supplies are all at risk due to climate change. The Intergovernmental Panel on Climate Change reported in its fifth assessment report that the global land surface temperature rose by 0.85°C between 1880 and 2012 and by 0.78 °C between 2003 and 2012, relative to values from 1850 to 1900 (IPCC, 2014b). Following projections, the global average temperature will rise by 1.4 to 5.8°C by 2100 (Noguer *et al.,* 2001). There is a growing discrepancy between past climate patterns and the current and future climate conditions brought about by human activity (Chuwah *et al.,* 2013).

Additionally, Verichev *et al.* (2020) proposed a set of four potential climate change scenarios known as Representative Concentration Pathways (RCPs). RCP 4.5. RF will stabilize at roughly 4.5 W/m^2 along an intermediate stabilization pathway after 2100. Geological storage, lower-emission energy sources, increasing electricity use, and CO_2 capture technologies will all be required to reduce emissions. In addition, there will be more forest land under this scenario than in the current one. In addition, it appears that CO_2 emissions from industrial and energy sources will rise until 2040 and fall to the recommended atmospheric CO_2 concentration of 538 parts per million in 2100. Every year is different as it involves natural and environmental disasters (Symanski *et al.,* 2021).

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Over the previous ten years, natural disasters claimed the lives of almost 60,000 people per year on average throughout the world (Wiranata and Simbolon, 2021). Consequently, the report estimates that 0.1% of deaths worldwide. The *Paris Agreement* builds on the Convention by uniting all countries for the first time behind a common goal to take bold action to stop climate change and prepare for its effects, along with more funds to support developing nations in their efforts. Enhancing the global response to the threat of climate change by limiting the rise in global temperature this century to well below 2°C over pre-industrial levels and pursuing efforts to limit the temperature increase to 1.5°C is the main objective of the Paris Agreement (Sharma *et al.,* 2020; Sharif *et al.,* 2020; Chien *et al.,* 2021).

Aiming to keep global warming this century well below 2°C over pre-industrial levels, the Paris Agreement calls for swift action in the energy, transport, infrastructure, and agricultural sectors, all while considering the need for these sectors to support an expanding human population (Wynes and Nicholas, 2017). Released in August 2021, the IPCC's Sixth Assessment Report by Working Group I reported that, over the previous forty years, every decade has been warmer than the previous one, with a 1.09°C increase in global mean surface temperature (GMST) from 2011 to 2020 compared to the pre-industrialization era (represented by the 1850–1900 average). However, in addition to its side effects on human comfort, the acceleration of global warming will also cause a marked increase in human discomfort, with the implications being more pronounced in low latitudes than in high latitudes (Li *et al*., 2017; Huang *et al.,* 2022).

2.5.2. Europe

Heat exposure significantly increases illness and death, which puts high-risk groups in Europe and around the world at serious risk (Ebi *et al.,*2021). Europe represents a significant climatic hotspot. As a result of a severe string of heat waves that caused temperature extremes, drought, and fire activity, the summer of 2022 was the warmest season on record in Europe (Rodrigues do Nascimento*,* 2023). Existing surveillance systems kept an eye on the summer of 2022's record-breaking temperatures, which prompted several national and local heat prevention and adaptation strategies. The EU-28 (which includes the UK, which is no longer a member of the EU) is expected to produce approximately 30% of its total agricultural greenhouse gas emissions by 2025 from dairy cows (including lactating dairy cows, dry dairy cows, heifers, and calves), according to projections made using the CAPRI (Common Agricultural Policy Regionalized Impact) model of the European Commission (EC, 2015). Specifically, increasing emissions of CH_4 and N_2O from manure management have a consequence of intensifying dairy production systems (Petersen, 2018).

Consequently, knowledge of the effects of mitigation efforts as well as the effects of climate change on cattle production is necessary for lowering GHG emissions sustainably (Chang *et al.,* 2021). Changes to manure storage procedures can, in this case, lower manure greenhouse gas emissions (Rojas-Downing *et al.,* 2017). According to Montés *et al.* (2013*),* these include reduced storage temperatures, shorter storage times, solid-liquid separation, and anaerobic digestion (AD). Compared to the 1.1°C increase from 1850 to 1990, the global mean temperature is predicted by climate models to rise by 1 to 5.7 °C by the year 2100 (IPCC, 2021).

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2.5.3. Spain

Extreme heat waves and severe summer droughts have become more common in Spain during the last 20 years (Espín-Sánchez and Conesa-García, 2021; Serrano-Notivoli *et al.,* 2022). The 2003 warm summers broke records for high temperatures (García-Herrera *et al.,* 2010). These summers are notable for being the first significant indication of European susceptibility to extreme heat events (Robine *et al.,* 2008; Russo *et al.,* 2015). Fifty-seven per cent of the nation's greater summer temperatures were recorded in the first 22 years of the twenty-first century and impacted hydroclimate conditions in addition to temperature.

Summers in the Mediterranean climate are often dry, but these warm conditions have had unprecedented effects on natural and socioeconomic systems; droughts such as those that occurred in 2012 (Machado-Trigo *et al.,* 2013; Tejedor *et al.,* 2016) and 2017-2019 (Sánchez-Benítez *et al.,* 2018; Kew *et al.,* 2019; Hoy *et al.,* 2020) changed the regular cycle of evapotranspiration rates (Rita *et al.,* 2020; Mathbout *et al.,* 2021). A unique combination of extreme weather events, such as a high frequency and intensity of heatwaves (HW) and a prolonged yet frequent period of low precipitation quantities across most of the territory due to an abnormal air pattern, have characterized Spain's summer of 2022.

Climate change is just one of Spain's many pressing issues. Energy is just one aspect of the problem. To meet the goals established by the European Union, which call for a 32.5% decrease in energy efficiency (EC) by 2030, Spain must cut EC to meet its energy efficiency and savings targets set by the European Union (EU, 2018). By 2050, this latter goal will help the Spanish economy become carbon- neutral (EU, 2019). But climate variables like temperature swings and the variety of climate zones can impact this difficulty (López-Ochoa *et al.,* 2021). So far, studies examining how local temperature perceptions affect beliefs about climate change have concentrated on the high and low ends of the temperature spectrum. These studies have shown that when temperatures are perceived as being warmer or colder than usual, beliefs about climate change rise (Hamilton and Stampone, 2013; Zaval *et al.,* 2014; Kirilenko *et al.,* 2015; Sisco *et al.,*2017). Regarding Spain in particular, maximum daily temperatures under the Representative Concentration Pathway (RCP) 8.5 emission scenario were expected to increase from a mean of 28.7°C during the summer months between 2000 and 2009 to 30.3°C between 2021 and 2050 and 33.6°C between 2051 and 2100 (Diaz *et al.,* 2019). These studies have demonstrated that milk production involves net GHG emissions (Prado *et al.,* 2013; Laca *et al.,*2020). The difficulty in finding an agreement between reducing greenhouse gas emissions and enhancing livestock output is due to ongoing climate change and industry demands (Chang *et al.,* 2021).

According to projections made by the IPCC in 2021, hydrological and agricultural/ecological droughts in the Mediterranean will worsen, and pluvial floods in Northern Europe are expected to rise.

2.5.4. Galicia

One of Europe's most prone regions to fires is Galicia, in Spain. In comparison to other land uses, such as croplands, the grasslands in Northern Spain have significant soil organic carbon (SOC) storage (Ganuza and Almendros, 2003), reaching more than 100 Mg (Rodriguez-Martin *et al.,* 2016). Nonetheless, various studies have assessed the carbon

footprint of grassland-based dairy production in Northern Spain under the current climate, primarily using the Life Cycle Assessment (LCA) framework. A study conducted in Northern Spain by Jebari *et al.* (2022) examined the regional estimate of net greenhouse gas emissions for grassland-based cattle production systems in moist temperate settings. To our knowledge, Northern Spain hasn't had any regional assessments of net GHG under climate change conditions. Therefore, the primary goal is to provide emission and sink estimates of the three top greenhouse gases (CH_4, N_2O, and CO_2) for dairy production systems in Northern Spain under various future climate projections and manure management scenarios at different scales (including the soil and grassland levels of barns).

In 2023, Galicia experienced its warmest and wettest year in the past six decades. The average temperature was 14.9°C, which is considered extremely high1. The winter of 2023-2024 was also notably warm, with an average temperature of 10°C, making it the fourth warmest winter on record since 1961.

In 2023, Galicia, like much of Europe, experienced significant consequences due to high temperatures. Some significant impacts were:

1. *Health Impacts:* The extreme heat contributed to a high number of heat-related deaths across Europe, with Spain being one of the most affected countries. Vulnerable groups, such as the elderly and those with pre-existing health conditions, were particularly at risk.
2. *Agricultural Stress*: The high temperatures and associated drought conditions put considerable stress on agriculture, affecting crop yields and livestock health.
3. *Wildfires*: The heatwave increased the risk of wildfires, which can cause extensive damage to forests, property, and wildlife.
4. *Water Shortages:* Prolonged periods of high temperatures led to reduced water levels in rivers and reservoirs, impacting water supply for both domestic and agricultural use.
5. *Economic Impact:* The combination of health issues, agricultural losses, and damage from wildfires and water shortages had a significant economic impact on the region.

These events highlight the importance of climate adaptation and mitigation strategies to cope with the increasing frequency and severity of heat waves.

In ruminants, heat stress significantly impacts methane emissions through different mechanisms:

1. *Altered Digestion*: Heat stress affects the rumen (the first stomach of ruminants) environment, leading to changes in microbial populations. This can result in altered fermentation processes, which may increase methane production
2. *Reduced Feed Intake*: Cattle tend to eat less when they are heat-stressed, which can lead to a decrease in overall methane emissions. However, the efficiency of feed conversion may also decrease, potentially offsetting this reduction
3. *Increased Respiration Rate*: Heat-stressed cattle have higher respiration rates, which can affect their metabolic processes and potentially influence methane emissions.
4. *Health and Productivity*: Heat stress can lead to health issues and reduced productivity in cattle, which indirectly affects methane emissions. Healthier, more productive cattle

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typically have more efficient digestion and lower methane emissions per unit of product (e.g., milk or meat).

Overall, the relationship between heat stress and methane emissions in cattle is complex and influenced by various factors, including changes in feed intake, digestion, and overall health.

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3. THE TIER PREDICTIVE MODELS

The organization responsible for verifying guidelines for GHG-emission analysis and reporting is the *Intergovernmental Panel on Climate Change* (IPCC). The establishment of the IPCC was approved by the UN *General Assembly* in 1988. Its original project, as briefed in UN *General Assembly Resolution* 43/53 of December 6, 1988, was to sketch an inclusive review and references concerning the state of support of the science of climate change (https://www.ipcc.ch/about/history/) the social and economic impact of climate change, and potential response strategies and elements for inclusion in a possible future international convention on climate.

Since 1988, the IPCC has conducted six assessment cycles and released six *Assessment Reports*, the most extensive scientific data about climate change produced worldwide. It has issued a variety of *Methodology Reports*, *Special Reports*, and *Technical Papers* in response to requests for specific scientific and technical matters from the *United Nations Framework Convention* on *Climate Change* (UNFCCC), governments, and international organizations (Ashworth and Clarke, 2023) (**Image 9**).

The *Intergovernmental Panel on Climate Change* (IPCC) is a sheet of 195 participant nations (https://www.moew.government.bg/en/climate/ipcc/about-ipcc). *The Panel meets in Plenary Sessions* on a yearly minimum. The *Sessions* are attended by hundreds of professionals and experts from relevant ministries, agencies, and research institutions from member countries and *Observer Organizations* (https://www.moew.government.bg/en/climate/ipcc/about-ipcc/). The *Panel* works by consensus to decide on the organization's budget and work program, the scope and outline of its reports, issues related to the principles and procedures of the IPCC, and the structure and mandate of *IPCC Working Groups and Task Forces*. The *Panel* also approves and adopts IPCC reports and determines the IPCC *Chair* and members of the IPCC *Bureau* and the *Task Force Bureau*. IPCC *Working Groups* hold *Plenary Sessions* to review the *Summary for Policymakers of Assessment and Special Reports* before they are considered by the Panel. View documentation from the IPCC and *Working Group Plenary Sessions* (https://www.ipcc.ch/documentation/ipcc-wg/).

Image 9. Global temperature rise projections. Sourced by (IPCC. https://www.ipcc.ch/).

The IPCC reports address various aspects of climate change, including the impact of livestock on the environment. Here are some key points from the IPCC reports related to livestock:

1. *Climate Change and Land*: *The Special Report on Climate Change and Land* discusses how supply-side practices, including livestock emissions reduction, can contribute to climate change mitigation1.
2. Agriculture: The AR4 report on Mitigation of Climate Change includes a chapter on agriculture that covers the role of livestock in greenhouse gas emissions2.
3. *Food Systems Transformation*: Recent IPCC climate reports highlight the urgency of transforming food systems, including the impact of livestock on water availability and food production3.
4. *Emissions Peak*: It is suggested that emissions from livestock must peak before 2025 in *High-Income Countries* to align with the *Paris Agreement* goals.

These reports emphasize the significant role of livestock in climate change and the need for sustainable management and practices to mitigate their impact.

3.1. RELEVANCE OF IPCC PREDICTIVE MODELS IN GALICIAN DAIRY LIVESTOCK

The European Union (EU) is a significant producer of milk and dairy products as part of the Common Market Organization (CMO). All EU countries produce milk, which accounts for a substantial portion of the value of EU agricultural production. EU milk production is estimated at around 155 million tons per year. The dairy cattle sector in Spain holds great economic importance as the third most significant livestock production sector, following the pig and beef sectors. Over the years, it has adapted to agricultural market challenges and foreign competition by integrating technological and structural advancements. This sector significantly contributes to maintaining the rural environment, economic diversification, and population settlement in areas where other economic activities may not be feasible. Additionally, dairy farms located in rural areas play a vital role in preventing depopulation, creating both direct and indirect employment opportunities, and revitalizing rural communities (European Commission, Agriculture and rural development, 2023).

The dairy cattle production industry is currently facing a significant challenge due to the rise in methane emissions attributed to enteric fermentation. These emissions, coupled with gases produced from the fermentation of cattle manure, contribute to the overall increase in greenhouse gases (GHGs). Studies indicate that the livestock sector accounts for up to 17% of total anthropogenic methane emissions globally. In Spain, a 2022 report from the Ministry for Ecological Transition and the Demographic Challenge (Ministerio para la Transición Ecológica y el Reto Demográfico Gobierno de España, 2022) revealed that in 2020, livestock's enteric fermentation contributed 16.085 kt CO2-eq in net emissions. This marked a 12.0% increase from 1990 and a 0.5% increase from 2019. **Table 3** illustrates the emission trends, categorizing the highest contributors as dairy and beef cattle.

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Table 3. Methane emissions (in kilotons CO_2-eq) from enteric fermentation cattle.

	1990	2005	2015	2019	2020
Dairy cattle	3397.1	2788.0	2642.7	2540.4	2528.4
Beef cattle	5581.3	8341.6	8352.0	9068.1	9121.4

Over the historical series, beef cattle have gained importance in the total category, from 39% in 1990 to 57% in 2020, at the expense of dairy cattle emissions (from 24% to 16%). These two herds, together with sheep, account for about 90 % of the total emissions of the livestock sector.

Methane has a relatively short lifespan of 12.2 years (Myhre *et al*., 2013). It is released alongside other gases during the digestive process in an animal's gastrointestinal tract, specifically the reticulo-rumen and intestine, and through manure methanogenic processes. It is currently recognized as a short-term climate enhancer. Despite its short atmospheric lifetime and lower emission quantities compared to CO_2, its heat-trapping potential is 28 times greater than CO_2 and N_2O (Islam *et al.,* 2022). There are differing views on the extent of livestock's contribution to global CH_4 emissions. Studies analyzing the isotopic composition of CH_4 in the atmosphere, ice cores, archived air, and data from bottom-up and top-down approaches suggest that the post-2006 surge in CH4 emissions is primarily driven by increases in microbial CH_4 (Lan *et al*., 2024) (**Figure 21**).

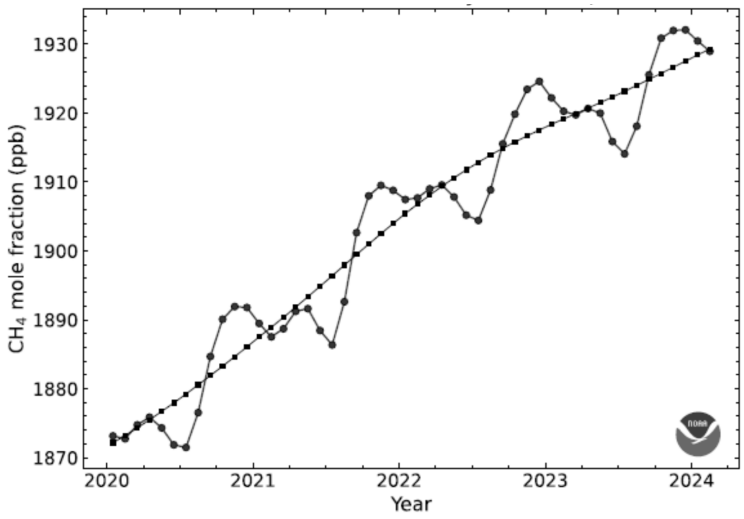

Figure 21. Global trend in CH_4. The line with circles is globally averaged monthly mean values centered on the middle of each month. The line and squares show the long-term trend (in principle, like a 12-month running average mean) From: Lan *et al.*, 2024.

Microbial emissions are due to methane-generating microbes (methanogens) found in anaerobic environments such as natural wetlands and rice paddies, oxygen-poor freshwater reservoirs (such as dams), digestive systems of ruminants, and organic waste deposits (such as manure, sewage, and landfills). This is in line with the report prepared by the Spanish Ministry

for Ecological Transition and the Demographic Challenge (2022), which considers that almost half of the emissions in the Spanish farming industry are generated using fertilizers and soil management, while the other half is caused by livestock farming (enteric fermentation and manure management). Ruminant species, mainly cattle, have the highest CH_4 emission rates, while poultry has almost negligible CH_4 release. On the other hand, monogastric animals like pigs fall between these two extremes, as the microbial density in their stomach and small intestine is limited.

Spain has a well-established legal framework for creating national emission inventories. The General Bureau for Biodiversity and Environmental Quality is the main authority responsible for the Spanish System of Inventory and Projections of Emissions to the Atmosphere, which monitors greenhouse gases and atmospheric pollutants.

However, the livestock sector has significant gaps in its inventory systems due to its unique characteristics, unlike other industries. Agriculture and livestock are considered less energy-intensive sectors. While many efforts have been made to measure and address issues in livestock management, the lack of established reference values, according to European Community regulations, leaves veterinary professionals unsure about how to determine these values for different field conditions.

When estimating greenhouse gases, there are many methods to choose from, each aimed at getting accurate and precise results for the emissions of each activity. These methods are meant to improve over time, moving towards more advanced approaches. The selection of the methodology falls into two types: 1) Methods based on observed emissions data; and 2) Methods based on calculation procedures that include emission factors (EF).

In the 2030 scenario, one proposed way to reduce GHG emissions is by controlling enteric fermentation through dietary modifications in the livestock sector. Currently, the carbon footprint of dairy farms is measured in kilograms of carbon dioxide equivalent per liter of milk (KgCO2/L), with 1 Kg of CO2 per liter of milk considered optimal. In France, the sector has introduced a differentiated label that identifies farms with emissions levels below 0.8 kg CO2 per liter of milk. Around 6.000 farms have already registered for the label, known as *Bas Carbone* (Cortiñas, 2017).

3.2. MEASURING ENTERIC METHANE IN CATTLE: CURRENT METHODS

In this context, we need to consider the role of cattle in anthropogenic gas emissions and the necessity of creating inventories of livestock CH_4 emissions on both a national and global scale. Sources of uncertainty in methane emissions from cattle include animal numbers, feed intake, diet composition, breed, Physiological stage and enteric methane emission intensity (Villanueva *et al.,* 2023. The most used techniques for estimating these emissions are respiration chambers, the SF6 tracer technique, and the automated head-chamber system (GreenFeed). All three methods have been used successfully in numerous experiments with

dairy or beef cattle in different controlled environmental conditions. However, studies comparing these techniques have reported inconsistent results. To improve the accuracy of predictions, the data sets utilized should cover a broad range of diets and production systems both regionally and globally (Hristov *et al.,* 2018).

Despite the varying precision of these techniques, two factors can make these methods impractical at times: 1) the potential stress placed on animals due to handling, which can alter the parameters being measured, and 2) the availability of farms for carrying out such measurements, especially if the goal is to conduct measurements on field conditions which doesn't have the controlled monitoring standards of research centers. In addition, not all livestock farmers would favor this kind of design, which could negatively affect productivity, even if it effectively contributes to reducing CH_4 emissions (Martin et al., 2010). There have been suggestions and applications of indirect methods to measure methane EF. These methods are associated with lower accuracy and greater uncertainty in the emission data compared to the direct methods mentioned above. Changes in metabolic activities, differences in feed efficiency, and variations in ruminal fermentation can all influence the amount of CO_2 produced by the animal, thus modifying the predicted methane emission (Huhtanen et al., 2015; Villanueva *et al*., 2023). However, it uses easily accessible on-farm parameters such as ration or animal characteristics like live weight and dry matter intake (DMI), or milk production (Jaurena et al., 2015).

DMI is a crucial factor in predicting CH4 emissions from livestock. Researchers have studied the relationship between measured CH_4 production with DMI and neutral-detergent fiber (NDF). It has been found that the correlation between CH4 emissions and DMI is strong when there is a wide range of DMI and weak when the range of DMI is narrower. The dairy NRC (2001) model uses the cow's metabolic body weight, milk yield, and stage of lactation to predict DMI.

Other studies have found that milk solids concentrations can be useful in predicting CH_4 emissions from dairy cattle. Moraes *et al*. (2013) highlighted the significance of milk fat content as a key variable for predicting CH_4 emissions. Furthermore, Kandel et al. (2014) discovered a moderate correlation between predicted CH_4 intensity (calibrated from SF6 tracer data) and protein yield. However, additional studies have found that attempting to correlate different milk components with CH_4 values obtained from direct methods may not be reliable indicators of CH_4 emissions (Negussie, 2017).

In the last ten years, researchers have been studying the use of milk fatty acids (MFA) as indicators of CH_4 emissions due to their direct connection to microbial digestion in the rumen. The mammary gland's de novo production of MFA primarily utilizes acetate (85% of de novo synthesized FA) along with β-hydroxybutyrate (10 to 15%) and a small amount of propionate to produce short-chain FA (C4, C6, and C8), most medium-chain FA (C10, C12, and C14), and about 60% of C16 (Chilliard *et al*., 2000; Couvreur, 2007).

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Numerous prediction equations have been developed to explain the relationship between milk fatty acids (measured using gas chromatography) and methane production from the digestive system. However, the correlation index has varied between 47% and 95%, and the links between individual milk fatty acids and methane production differ significantly (Van Gastelen and Dijkstra, 2016). These differences may stem from the direct methane measurement technique and the way methane emissions are expressed. Furthermore, Vanrobays *et al.* (2016) discovered the correlations between methane emissions and MFA change throughout lactation, providing further insight into the diverse findings in the literature.

To evaluate CH_4 emissions in on-farm settings, it is essential to utilize indirect methods that are suitable for large-scale applications. This is increasingly crucial due to the necessity of accurately determining GHGs emissions in diverse farming environments. Moreover, there is a rising requirement for the establishment of standardized baseline values to serve as a reference for implementing corrective strategies based on nutrition. The use of advanced statistical programs allows for the collection of reliable data related to animal health and productivity. These critical points enable specialists to take prompt and effective actions.

The GLOBAL NETWORK project (Global Network for the Development and Maintenance of Nutrition-Related Strategies for Mitigation of Methane and Nitrous Oxide Emissions from Ruminant Livestock 2014-2018) is an international collaborative initiative of animal scientists from all continents except Africa (Niu et al., 2018). The database contains records of enteric CH_4 production, along with corresponding data on DMI, body weight (BW), GEI, crude protein (CP), ethereal extract (EE), NDF, and ash. Additionally, it includes information on milk yield (MY) and concentrations of milk fat and crude protein. With these parameters and depending on the procedure used (direct or indirect) for the measurement of methane emissions, different statistical models have been developed, with great variability in the results. In addition, studies (Villanueva et al., 2023) indicate that although there are no significant differences in enteric CH_4 emissions between breeds of dairy cows with different genetic backgrounds, those with more *Bos taurus* genes present higher CH_4 emissions. Therefore, *B. taurus x B. indicus* genetic crosses should be considered for future trials, especially in the tropics.

In this context of uncertainty, and from a clinical point of view, the predictive equations developed by the International Panel of Climate Change (IPCC) in its various updates are emerging.

3.3. THE TIER PREDICTIVE MODELS FROM THE IPCC

The Intergovernmental Panel on Climate Change (IPCC) was established in 1988 by the United Nations Environment Programme (UNEP) and the World Meteorological Organization (WMO). The United Nations General Assembly endorsed its creation that same year. The IPCC's mission is to provide policymakers with regular scientific assessments on climate

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change, covering topics such as the science of climate change, its social and economic impact, and potential response strategies. Since its inception, the IPCC has produced six comprehensive Assessment Reports and several other specialized reports, contributing significantly to international climate policymaking (https://www.ipcc.ch/about/history/). Since then, the IPCC has played a key role in institutionalizing this concept and is recognized as the leading authority on global climate science (Allan, 2017; Beck and Mahony, 2018).

The IPCC reports address various aspects of climate change, including the impact of livestock on the environment. Here are some key points from the IPCC reports related to livestock such as agriculture procedures or the significant role of livestock in climate change and the need for sustainable management and practices to mitigate their impact. Suggests that emissions from livestock must peak before 2025 in high-income countries to align with the Paris Agreement goals.

3.3.1. TIERS and methane emission factors (EF-CH_4 kg CH_4 $head^{-1}$ $year^{-1}$) calculations

The IPCC guidelines classify the methodological complexity into different TIERS (or levels) for estimating emissions of each greenhouse gas in mass units (**Figure 22**). These TIERS are based on the amount of data required and the level of analytical complexity (Chen and Brauch, 2021). TIER 1 is an empirical method that uses default EFs per head of livestock to calculate enteric CH_4 emissions. TIER 2 is an enhanced method that requires information about animal categories, feeding, and production systems. The TIER 3 approach is used when a country-specific methodology for enteric CH_4 emission estimation has been developed by the IPCC (2019).

TIER-2 and TIER-3, known as higher tier, are generally considered more accurate than the default Tier-1 approach due to their suitability for specific applications (Jo *et al*., 2016; Booysen and Kleingeld, 2018; Gitarskiy, 2019). The TIER 2 method is recommended for countries with large livestock populations, using mostly national parameters related to feed diet, production characteristics, energy requirements, and energy/protein ratio. The precise data for each level of measurement or tier are shown in **Figure 22**. The IPCC in its various reports encourages the development of studies in different countries and management conditions to increase satisfactory inventories. Unfortunately, to our knowledge, very few studies have been carried out in Spain on this topic.

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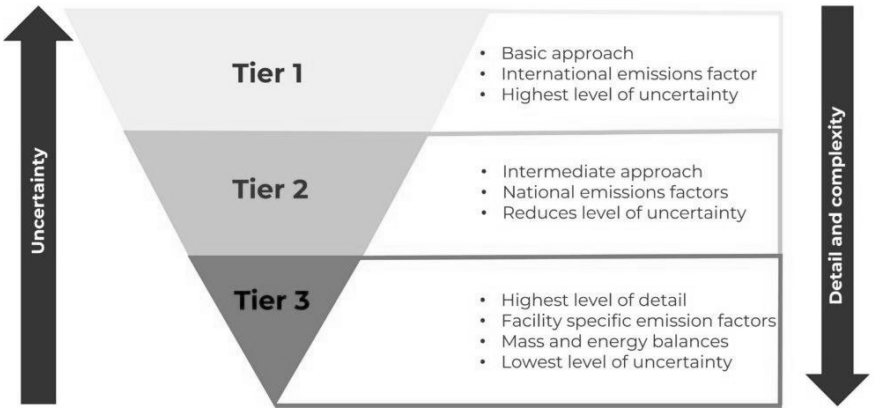

Figure 22. Tier methodological complexity. Available at: https://www.agrecalc.com/home

The enteric methane conversion factor (Ym%) is a crucial specific value in these models. It provides accurate data on the inventory of milk yield (MY). This value is related to the percentage of gross energy intake (GEI) and CH_4 production, considering the production system, type and age of livestock, and characteristics of feed available on the farm within a reasonable range of uncertainty (Jaurena *et al*., 2015; Ibidhi *et al*., 2021). Ym (%) is defined as the percentage of energy provided with the ration that is converted into CH_4 (Ibidhi *et al*., 2021). The energy provided with the ration that is converted into CH_4 depends on several factors such as animal production, as well as the quality and digestibility of the different diets, taking as a reference the total mixed ration (TMR) with an inverse relationship between Ym (%) and digestibility (Cambra-López *et al*., 2008). The calculation of the emission factor directly depends on Ym. Due to the high level of uncertainty in estimating Ym, it is relevant to improve the accuracy of this parameter in future studies. Researchers can determine site-specific Ym values through more thorough studies in areas where a Tier 3 approach is available, following IPCC guidelines. In regions where this level of information is not available, Tier 2 and the estimated Ym (%) can be used (Jaurena *et al.,* 2015).

The IPPC (2006, 2019) has developed recommendations for the Ym factor, considering milk production, the level NDF in the diet, and their digestibility percentage. In cases where country- or region-specific methane emission factor (Ym %) values are unavailable, it is viable to utilize estimations put forward by various studies (Appuhamy *et al*., 2016; Jayasundara *et al*., 2016; Niu *et al*., 2018) that have contributed to the IPPC Refinement (2019) for CH_4-EF calculations. Recent studies have also adopted these assumptions (Niu *et al*., 2021). All that is needed is to know in which subcategory the studied cows fall based on production, productive status, and feed quality (focused on digestibility, DE %) and NDF (% DMI). These references are listed in **Table 4**

Once the subcategory is selected, CH_4-EF- for each dairy cattle group can be calculated using the IPCC Tier 2 approach based on gross energy intake (GEI) and Ym (%) as follows:

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$$\underline{EF = [GEI \times (Ym/100) \times 365 \text{ days})]}$$

$$55.65 \text{ MJ/kg } CH_4$$

Where EF is the CH_4 emission factor (kg CH4/head/year) and Ym represents the CH_4 conversion rate (%), which is the fraction of gross energy (GEI) in feed converted to CH_4. The factor 55.65 (MJ/kg CH_4) is the energy content of methane.

The GEI value is developed by several mathematical equations that consider factors such as energy for maintenance, pregnancy, lactation, and so on… such complicated calculations are now carried out by sophisticated computer software designed to manage the information and predict the nutrient requirements and supply in each production situation, providing GE values according to the diet applied and production. Veterinary nutritionists are now present on every farm to assess the quality of diets and ensure they meet production requirements. Utilizing the appropriate software platform is crucial for precise feed assessment, a key component of smart farming. In doing so, we aim to address the concerns of veterinarians, who are not nutritionists but are responsible for managing livestock farms, including the control of CH_4-EF.

Table 4. Cattle methane conversion factor (Ym, %) in different conditions and diets (IPCC, 2019)

Livestock category	Description	Feed quality digestibility (DE %) and NDF (% DMI)	Ym (%)
Dairy cows	High-producing cows (>8500 kg/head/year)	DE ≥ 70 NDF ≤ 35	5.7
	High-producing cows (>8500 kg/head/year)	DE ≥ 70 NDF ≥ 35	6.0
	Medium-producing cows (5000-8500 kg/head/year)	DE → 63-70 NDF > 37	6.3
	Low-producing cows (< 5000kg/head/year)	DE ≤ 62 NDF > 38	6.5
Non-dairy and multi-purpose cattle	> 75% forage	DE ≤ 62	7.0
	Rations of 75% high quality forage and or mixed rations	DE → 62-71	6.3
	Feedlot (all other grains 0-15% forage)	DE ≥ 72	4.0
	Feedlot (steam-flaked corn, ionophore supplement, 0-10% forage)	D E> 75	3.0

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OBJECTIVES

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Based on the information provided and the wide data registered by different studies or international reports, precise data analysis from dairy cattle farms is an essential tool today that will allow the veterinarian to decide to reduce the Greenhouse Gas Emissions Factors within the national/international context.

Therefore, the **GENERAL** objective of the study is to develop a TIER-2 model at on-farm conditions based on the data provided by the different dairy production systems in Galicia to create a GHG inventory, within the framework of the International Standards and considering its varied production system.

To achieve this objective, the following **SPECIFIC** objectives are set out:

1. To determine methane emission values in high-production dairy farms assuming the Ym value (%) recommended by the *2019 Refinement to the 2006 IPCC Guidelines*.
2. To evaluate the changes that EF showed depending on whether the animals are at their maximum milk yield (peak lactation) or in the dry-off period.
3. To explore the effect that the diet supplied may have on the CH_4-EF results achieved.
4. To evaluate, at on-farm conditions, which physicochemical components of the ration could contribute to lower emissions of methane.
5. To assess the effects of lipid supplementation on the main physicochemical components of milk

We would furthermore like to point out that to the best of our knowledge, this is the first study carried out in Spain on dairy cattle that considers the recommendations of the 2019 Refinement to the 2006 IPCC Guidelines. This institution emphasizes the importance of conducting studies that focus on management to carry out precise analyses for establishing more refined predictive models as a reference.

With this purpose, this research was carried out in commercial dairy facilities that are standard models within the Galician dairy industry and not in a research center whose results cannot always be extrapolated to field conditions.

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GENERAL MATERIALS AND METHODS

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1. EXPERIMENTAL DESIGN

1.1. CHARACTERISTICS OF MILK PRODUCTION IN GALICIA.

Galicia is one of the most important regions in Spain in terms of dairy production, standing out both for the quantity and quality of its milk. Here are some of the most relevant characteristics:

- *High Production*: Galicia produces approximately 40% of all the milk in Spain. This makes it the leading milk-producing region in the country.
- *Nutritional Content:* Galician milk is characterized by its high-fat content, averaging 3.89%, which is higher than the national average of 3.77%. In terms of protein, it matches the national average at 3.26%.
- *Cattle Breeds:* The Friesian breed is the most common in Galicia due to its high productivity and adaptation to intensive production. Other breeds such as the Brown Swiss, Montbéliarde, Fleckvieh, and Jersey have a residual presence.
- *Farm Structure:* Most farms are family-owned and medium-sized, with an average of 50 cows per farm. These farms are usually well-managed and equipped with modern technologies to improve efficiency and production quality.
- *Quality Control:* Farms in Galicia are subject to monthly production and quality controls. Milk samples are taken from each cow to analyze parameters that determine milk quality and animal health.
- *Innovation and Sustainability:* In recent decades, the Galician dairy sector has undergone significant modernization with the introduction of new dairy breeds and improvements in machinery and facilities. Additionally, there is a growing focus on sustainability and adopting ecological practices.

In Galicia, the livestock population is structured into two types according to the Ministry of Agriculture, Fisheries, and Food (MAPA) in the report issued in 2020:

Farmers with deliveries: They supply their production to a dairy processing plant. These tend to be intensive production systems.

Farmers with direct sales: Those who allocate all or part of their production directly to the final consumer, or those who produce and sell dairy products on the farm, where Galicia stands out as a leading autonomous community. These are usually organic dairy products.

1.2. ASPECTS OF THE STUDY AREA

The study for the development of this Doctoral Thesis was conducted by selecting various high-yield dairy cattle farms located in the provinces of Lugo and Pontevedra (Galicia). These farms belong to the first group mentioned; therefore, their production was intended for sale to dairy processing plants.

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The studied farms are reflected in the following map (**Figure 23):**

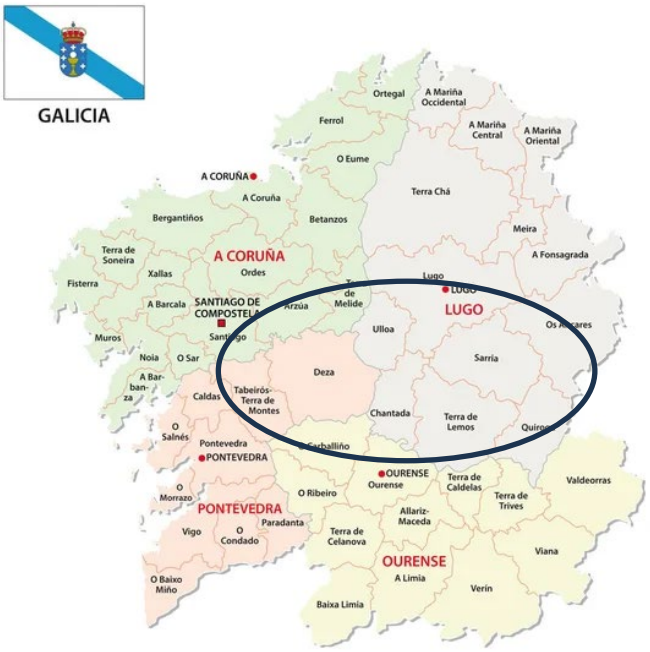

Figure 23. Area where the studied farms were located.

1.2.1. Agricultural Characteristics

1.2.1.1. County of Deza

The Comarca del Deza, located in the north of the province of Pontevedra, is known for its rich agricultural activity. Some of its main characteristics include:

Agricultural and Livestock Production: The region stands out for its high agricultural and livestock productivity, with a large useful agricultural area. The farms are usually intensive and well-managed, with investments in improvements and land consolidation.

Terrain and Climate: The region is bathed by the Deza and Arnego rivers, providing fertile soil suitable for agriculture. The climate is temperate and humid, ideal for the cultivation of pastures and forage.

Agricultural Protection: The Xunta de Galicia has declared the rustic land of agricultural protection in Deza as an area of special agricultural interest, which favors land mobility and prevents its abandonment.

1.2.1.2. South of the Province of Lugo

The south of the province of Lugo is also a region with a strong agricultural tradition. Some of its characteristics include:

Diversity of Farms: The region has a mix of agricultural and livestock farms, including both intensive and organic productions.

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Natural Conditions: The area benefits from mountainous terrain and fertile valleys, with a climate that favors the production of milk and other agricultural products.

Innovation and Sustainability: There is a growing focus on sustainability and innovation, with many farms adopting ecological practices and modern technologies to improve efficiency and reduce environmental impact.

These characteristics make both regions key areas for agricultural production in Galicia, significantly contributing to the local and regional economy.

1.2.2. Characteristics of dairy cattle facilities

Dairy cattle facilities in Galicia are designed to maximize productivity, animal welfare, and operational efficiency. Here are some of the most important features:

Design and Structure:

- ***Materials***: Predominantly use concrete for structures, fiber cement for roofs, and concrete block enclosures.
- ***Ventilation***: High ceilings with a minimum slope of 20% and opening systems that allow hot air to escape through the ridge, promoting good air circulation.
- ***Lighting****:* Natural lighting is essential, with strategically placed windows and openings to maximize sunlight and reduce the need for artificial lighting.

Rest Areas:

- ***Comfort****:* Rest areas should be comfortable, clean, and dry, with ample bedding that allows cows to lie down and get up without difficulty.
- ***Floor Materials****:* Smooth but non-slip floors to prevent injuries and improve animal comfort.

Feeders and Waterers:

- ***Accessibility***: Feeders and waterers should be easily accessible to all cows, distributed in a way that minimizes stress and competition among animals.
- ***Hygiene****:* It is essential to keep these elements clean to ensure the health and well-being of the livestock.

Milking Parlor:

- ***Efficiency***: Milking parlors are designed to maximize process efficiency, with modern equipment that ensures quick and effective milking.
- ***Animal Comfort***: The welfare of the cows during milking is prioritized, with facilities that minimize stress and facilitate handling.

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Environmental and Hygienic Conditions:

- ***Temperature Control****:* Ventilation and insulation systems that maintain an adequate temperature inside the facilities, protecting cows from extreme conditions.
- **Cleanliness**: Regular cleaning and disinfection procedures to prevent diseases and maintain a healthy environment.

Innovation and Sustainability:

- ***Technology***: Use of advanced technologies to monitor the health and production of cows, including sensors and data management systems.
- ***Ecological Practices****:* Adoption of sustainable practices, such as proper waste management and energy efficiency, to reduce environmental impact.

These characteristics ensure that dairy farms in Galicia are productive, sustainable, and respectful of animal welfare as can be seen in **Images 9 and 10**

Images 9 and 10. Example of one of the farms where the study was carried out.

1.3. EXPERIMENTAL FARMS SELECTION.

1.3.1. Identification of animal subcategories and breeds

The *2019 Refinement to the 2006 IPCC Guidelines*, in Chapter 10 establishes that "for countries with distinct regional differences, it may be useful to designate regions and then define categories within those regions. Regional subdivisions may be used to represent differences in climate, feeding systems, diet, and manure management."

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The dairy cattle population is reported by the Spanish Ministry of Agriculture, Fisheries and Food (https://www.mapa.gob.es) as an aggregation of animals based on their breed (native or foreign) Regional (autonomous) Communities, and age: milking cows, corresponding to animals aged >2 years; heifers, representing animals 1-2 years of age; and growing animals, corresponding to animals aged 2 years.

It is important to note that these farms mainly work with Holstein cattle. The number of animals of other breeds is less than 2%, and it is worth mentioning that there are no native breeds in Spain for milk production. Holstein has been the breed used since the beginning of dairy production in the country. Other breeds such as the Alpine Brown, the Montbeliard, the Fleckvieh, or the Jersey are only used minimally. The milk produced in Galicia has the highest fat content in Spain, with 3.89% compared to the average of 3.77%, a rate that is not surpassed by any other Autonomous Community. As regards protein, it is on the national average, with 3.26% (Fouz, 2018).

1.3.2. Selected farms, animals, husbandry and nutrition

We assessed twenty-one Holstein Frisian dairy farms that housed an average of 40 to 90 cows 136 each. We selected these farms based on the *2019 Refinement to the 2006 IPCC Guidelines* for 137 high-producing dairy cattle (>8500 Kg/head/year) that receive diets with over 70% feed quality digestibility (DE%) according to the formula DE (MJ/kg)/GE (MJ/kg) x100 and less than 35% NDF (DMI basis). These high-producing cows have calved at least once and are used principally for milk production.

Based on milk yield, we selected 9 farms with an average production between 38-40 Kgs of milk/cow/day, generating a mean annual output (at 305 days) within 11.590-12.200 Kgs of milk/head/305-day lactation standard (Group-A; G-A). This cohort comprises a total of 630 multiparous dairy cows (700 ± 50 kg) with a mean dry matter intake (DMI) between 27.3 Kg/day in peak lactation (day 150 after calving) and 11.7 Kg/day in the dry-off period (60 days before calving).

We also select 12 farms with an average production ranging from 32-35 Kgs of milk/cow/day, generating a mean annual production (at 305 days) between 9.800-10.500 Kgs of milk/head/305-day lactation standard (Group-B; G-B). This cohort comprises 900 multiparous dairy cows (700 ± 50 Kg) with a mean dry matter intake (DMI) of 22.6 Kg/day during peak lactation and 10.5 Kg/day during the dry-off period. Overall, this study comprises 1530 dairy cows from two high-production groups.

The farms were selected considering similar management conditions to ensure applicable data for this type of exploitation and based on an intensive free-stall housing system and diet formulation.

The diet after calving and during the lactation period consisted of a base ration, fed as a daily total mixed ration (TMR) comprising corn silage, grass silage, and a specific concentrate composed of corn, rapeseed meal, soybean flour, barley, and beetroot molasses. In 157 the dry-off period, the diet was comprised mainly of corn silage, grass silage, and a commercial concentrate composed of rapeseed meal, barley, and beetroot molasses, according to National Research Council (NRC, 2001) recommendations. Table 1 displays the mean values of total concentrate and forages at each stage for both groups.

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Diet was fed *ad libitum* three times a day at 07:00, 14:00 and 20:00 h. TMR samples were collected at the beginning of each stage and sent for chemical analysis to the reference laboratory for all farms (Aira SCG, Lugo https://www.aira.es) using the NDS-Pro software platform (RUM&N Company, Italy RUM&N (https://www.rumen.it), developed in collaboration with the Cornell Department of Animal Science and based on the NRC (2001) standards. The platform is an evolving application and it is continuously updated, as the results of new research are available, and as the updates of the biological model are released by Cornell University.

The NDS Professional software platform is founded on the dynamic model CNCPS (Cornell Net Carbohydrate and Protein System) v6.5/v6.55, a comprehensive tool for predicting nutrient requirements and supply in various animal production scenarios. NDS Professional employs technology licensed from the Department of Animal Science at Cornell University and is designed to support nutritional consultants, extension agents, and researchers in the formulation and evaluation of animal diets. Key features of NDS Professional comprise:

- *Nutrient Prediction:* This feature enables precise predictions of nutrient requirements and supply for diverse animal species.
- *Diet Formulation*: This functionality facilitates the formulation of novel rations based on existing feeds, aligning with established nutritional guidelines.
- *Ration Evaluation:* This component assesses the performance of rations after feeding, facilitating the evaluation of target performance.
- *Cost Optimization*: This feature compares different ration options to minimize feed costs and nutrient excretion.
- *Custom Feed Design*: This functionality allows for the design of customized feeds and premixes for feed mills.
- *Data Management*: NDS Professional employs a dual data management system comprising a master database for efficient data management and individual recipe files for facilitating data exchange between users.

CNCPS is widely recognized for advancing ruminant nutrition by providing accurate predictions of nutrient requirements and guiding effective feeding programs for improved animal health and productivity.

The Cornell Net Carbohydrate and Protein System (CNCPS) model is a comprehensive tool for predicting nutrient requirements and feed utilization in ruminant animals. It integrates various biological, chemical, and physical processes to provide a detailed assessment of nutritional needs. The key components considered in the CNCPS model include:

- *Feed Composition and Digestion*: Carbohydrate and protein fractions: The model analyzes different fractions of carbohydrates (NDF) and proteins based on their digestibility rates in the rumen and intestines.
- *Digestion and passage rates*: These rates influence nutrient availability and microbial growth.
- *Rumen microbial activities*: Microbial growth and efficiency: The model predicts microbial yield based on carbohydrate digestion and nitrogen availability.
- *Rumen Fermentation*: This process affects nutrient supply and utilization.
- *Animal physiology and requirements:* Tissue Requirements: Predictions include maintenance, growth, pregnancy, lactation, and body reserve changes. The model

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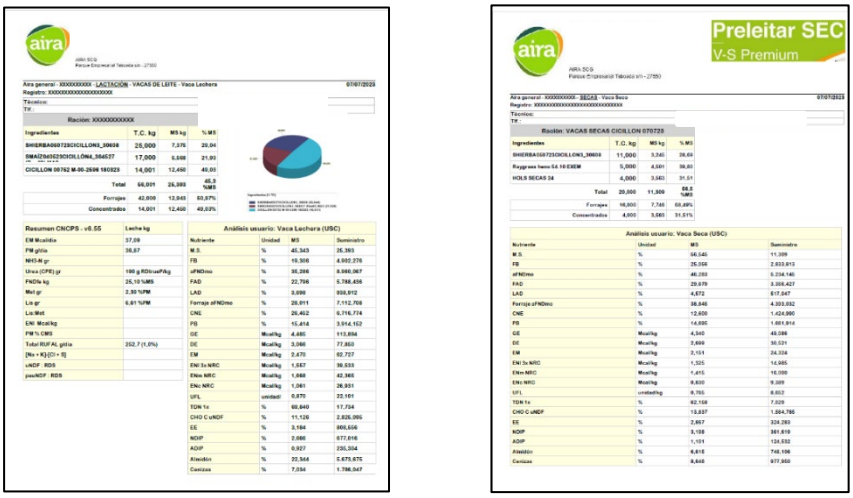
estimates nutrient supply from feed and microbial sources to meet animal requirements.

- *Environmental and Production Factors:* Forage Quality and Dietary Composition: These factors significantly impact nutrient supply and utilization. Dry Matter Intake (DMI): Accurate DMI is crucial for predicting nutrient requirements and supply.
- *Nutrient Excretion and Environmental Impact:* Nitrogen and phosphorus excretion. The model helps in minimizing these to reduce environmental impact.

By integrating these components, the CNCPS model provides a detailed framework for optimizing ruminant nutrition and reducing environmental impacts associated with animal production.

The chemical composition of diets during peak lactation and dry-off periods is shown in **Images 11 and 12**



Images 11 and 12. The chemical composition of diets during peak lactation and dry-off periods adopted in this PhD study.

In these diets, the aspects that were incessantly required were:

- Forage/concentrate ratio
- Dry Matter Intake (DMI)
- The amount of Crude Fibre (CF)
- Neutral Detergent Fiber on an Organic Matter basis (ANDFom)
- Acid Detergent Fibre (ADF)
- Lignin content of forage (ADL)
- The amount of Crude Protein (CP)
- Non-Structural Carbohydrates (NSC)
- Ether extract (EE)
- Ash level (Ash)

Milk production records were collected by the Friesian Breeders Association of Lugo (AFRICOR https://www.africorlugo.com). This legally authorized company recorded the daily yields of the animals previously identified with the farm code, bovine identification code, and calving date. In this way, it was possible to establish the total milk yields of the animals.

Dairy Control is a tool for farm management. The technicians who visit the farms collect the data, which, once processed, is returned to the farmer in a format that allows him to use this information in managing the farm.

This is the Association's main activity and is official, as it forms part of the Galician Dairy Control Centre (CEGACOL). This body coordinates the implementation of Official Dairy Control in Galicia. Control in Galicia.

Field technicians visit each of the collaborating farms once a month, during milking, taking note of the production of each animal and taking a sample of each animal. Production of each animal and taking a sample of milk from each of them for a sample of milk from each animal to be sent to the laboratory, where the analysis of fats, proteins analysis of fats, proteins, somatic cell count, lactose, fatty acids, and BHB (beta-hydroxybutyrate, an indicator of ketosis) and urea. of ketosis) and urea. Data are also collected during these visits.

Reproductive data such as calving dates, incidences, inseminations, and census (registrations and withdrawals) are also updated since the last visit. During the visit itself, a report of the work carried out is issued, containing indices and ratios calculated from the data collected. Each visit is carried out at an interval of between 26 and 35 days from the previous month's visit, and the month is always changed.

All the information is collected on mobile devices and processed in AFRICOR Lugo, where reports are generated and sent to the farmer as a working tool for better management of his farm. This information is stored in COLEWEB, the website of the Official Milk Control of Galicia, https://cegcol.xunta.gal

AFRICOR Lugo is ISO 9001-2015 certified and is the first dairy control association in Spain to obtain this accreditation (**Image 13**

Bureau Veritas Certification

Certificación

Concedida a

AFRICOR LUGO - ASOCIACION PROVINCIAL DE GANDEIROS DE LUGO PARA O CONTROL DE RENDEMENTOS

RONDA DE FINGOI, 117 - BAJO - 27002 - LUGO - ESPAÑA

Bureau Veritas Certification certifica que o Sistema de Xestión de dita Organización foi auditado sendo conforme coas esixencias da norma:

NORMA

ISO 9001:2015

O Sistema de Xestión aplícase a:

CONTROL LEITEIRO OFICIAL. TESTAXE DE EQUIPOS. TRAMITE E ENVIO DE DOCUMENTACIÓN AO LIBRO XENEALÓXICO. XESTION DE TOMA DE MOSTRAS PARA PROBAS DE FILIACIÓN POR ADN.

Número do Certificado:	ES129953-I
Data de certificación inicial con otra entidade de Certificación:	2007
Auditoría de certificación / renovación:	16-05-2022
Expiración do ciclo anterior:	16-05-2022
Certificado en vigor:	20-05-2022
Caducidade do certificado:	16-05-2025

Este certificado está suxeito aos termos e condicións e servizos de certificación específica

Bureau Veritas Iberia S.L.
C/ Valportillo Primera 22-24, Edificio Caoba, 28108 Alcobendas - Madrid, España

ENAC CERTIFICACIÓN

Image 13. AFRICOR Lugo ISO 9001-2015 certification.

The quality management system implemented by the Association complies with the requirements of the ISO 9001:2015 standard and seeks to develop working procedures that result in a better quality of service provided to members and customers.

2. DEVELOPMENT OF TIER-2 ENTERIC MODEL.

Considering the estimations proposed by different studies (Appuhamy *et al.* 2016; Jayasundara *et al.* 2016; Niu *et al.* 2018) which have contributed to the IPPC Refinement (2019), we assume a Ym (%) value of 5.7 for lactating cows. For cows on each farm in the dry-off phase, the Ym (%) value will be 6.3. Later studies have also followed these assumptions (Niu *et al*. 2021).

The CH_4-EF for each dairy farm (G-A and G-B) were developed using the TIER-2 approach based on gross energy intake (GEI) and Ym (%) as follows: (*Equation 10.21 from the 2019 Refinement to the 2006 IPCC Guidelines*):

$$\underline{EF = [GE \times (Ym/100) \times 365 \text{ days})]}$$

$$55.65 \text{ MJ/kg CH4}$$

where EF is the CH_4 emission factor (kg CH_4/head/year), and GE is gross energy intake (MJ/ head/day). The factor 55.65 (MJ/kg CH4) is the energy content of methane.

The calculation procedures are comprised of four steps: firstly, the net energy (NE) requirements for the different functions (maintenance, lactation, gestation, and growth) are calculated; secondly, the NE requirements are converted into gross energy (GE) requirements; thirdly, the CH_4 energy production is estimated using Ym (%) as a proportion of GE intake; and finally, the CH_4 energy production is converted into CH_4 emissions. To calculate gross energy (GE) by IPCC guidelines, activity data are required. The impact of input data on methane (CH_4) emission factors (EFs) was determined using the random forest procedure, which was employed with SAS software (version 9.4). The equation used to calculate the daily greenhouse gas (GHG) emissions of the dairy cattle subcategories is as follows:

$$GE = \left[\frac{\left(\frac{NE_m + NE_a + NE_l + NE_p}{REM} \right) + \left(\frac{NE_g}{REG} \right)}{\frac{DE\%}{100}} \right]$$

where, GE, Gross energy (MJ/head/ day); NE_m, Net energy for maintenance (MJ/day); NE_a, Net energy for activity (MJ/day); NE_l, Net energy for lactation (MJ/day); NE_p, Net energy for pregnancy (MJ/day); NE_g, Net energy for growth (MJ/day); DE%, Digestible energy expressed as a percentage of gross energy; REM, Ratio of net energy available in diet for maintenance to digestible energy consumed; REG, Ratio of net energy available for growth in a diet to digestible energy consumed. The following flow chart (Figure 24) presents the calculation of enteric methane emission factors for dairy cattle using the IPCC Tier-2 approach.

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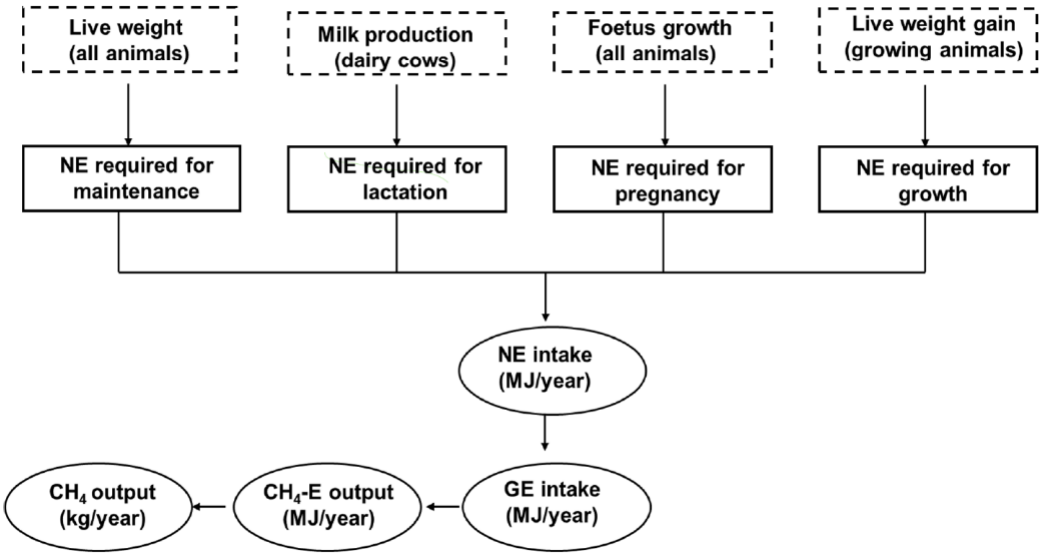

Figure 24. Flowchart of enteric methane emission factors for dairy cattle using the IPCC Tier-2 approach. The following abbreviations are used throughout this text: NE: net energy; GE: gross energy; CH_4: methane; CH_4-E: energy emitted by methane. (Ibidhi *et al.*, 2021)

The equations used to estimate NE requirements (NE_m, NE_l, NE_p, and NE_g), REM and REG are listed as follows, using the IPCC TIER-2 approach:

$$NE_m = Cf_i \times (\text{weight})^{0.75}$$

where, NE_m, Net energy for maintenance (MJ/day); C*fi*, maintenance coefficient.

$$NE_l = \text{Milk} \times (1.47 + 0.40 \times \text{Fat})$$

where, NE_l, Net energy for lactation (MJ/day).

$$NE_p = C_{pregnancy} \times NE_m$$

where, NE*p*, Net energy for pregnancy (MJ/day); $C_{pregnancy}$, pregnancy coefficient; NE_m, is Net energy for maintenance (MJ/day).

$$NE_g = 22.02 \times \left(\frac{BW}{C \times MBW}\right)^{0.75} \times WG^{1.097}$$

where, NEg, Net energy for growth (MJ/day); BW, body weight (kg); C, coefficient with a value of 0.8 for females; MBW, mature body weight (kg); WG, average daily weight gain (kg/day).

$$REM = \left[1.123 - \left(4.092 \times 10^{-3} \times DE\right) + \left(1.126 \times 10^{-5} \times (DE)^2\right) - \left(\frac{25.4}{DE}\right)\right]$$

where, REM, Ratio of net energy available in diet for maintenance to digestible energy consumed; DE, Digestible energy expressed as a percentage of gross energy.

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$$\mathrm{REG} = \left[1.164 - \left(5.16 \times 10^{-3} \times DE\right) + \left(1.308 \times 10^{-5} \times (DE)^2\right) - \left(\frac{37.4}{DE}\right)\right]$$

where, REG, Ratio of net energy available for growth in a diet to digestible energy consumed; DE, Digestible energy expressed as a percentage of gross energy.

TMR is the typical feeding system for dairy cattle production on intensive farms in Galicia, with animals fed in stalls. The dry matter intake (DMI) of dairy cattle was estimated from the daily was estimated from the daily nutrient requirements. For the study animals, DMI was determined based on average milk production and milk fat levels. No data were available on the DE (%) of TMR. It was assumed that the DE was based on the total content (%) of the TMR feed and the DMI (kg) of each study group. The process of assuming DE (%) is shown in **Figure 25** and milk fat content (%) through the dairy control.

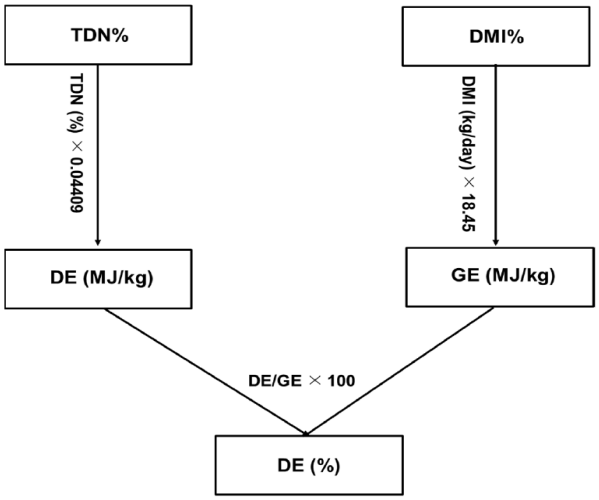

Figure 25. Flowchart of the feed digestibility assumption. TDN, total digestible nutrients; DMI, dry matter intake; DE, digestibility; GE, gross energy

As a new aspect in this work and given that the IPPC itself recognizes a certain degree of uncertainty in its predictive equations, we wanted to provide, beyond general values, more precise data, based on the productive phases. Therefore, for each stage, we replaced the value 365 (overall value) with the corresponding number of days (150 for lactation peak and 60 for dry-off) seeking a reliable and precise calculation of the EF. This approach agrees with the criteria of Ricci *et al*. (2013) who consider that in addition to dietary characteristics, the physiological stage of the animal is necessary to improve the prediction of CH_4 emissions.

The collected data conducted in this report was approved by the Animal Care and Use Committee of the University of Santiago de Compostela, according to the Spanish Regulations (RD 53/2013, legal provision number 1337) and the European regulation of animals for scientific purposes (Council of Europe, ETS no.123).

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RESULTS

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CHAPTER 1.

Estimating methane emissions from dairy cows in Galicia (N.W. Spain) during lactation and dry-off periods.

1.1. INTRODUCTION

Methane (CH_4) production in ruminants is a critical focus of veterinary research due to its link to various diseases (Johnson and Johnson 1985). On the other hand, livestock farming has become a major source of anthropogenic greenhouse gases, especially methane. However, the complexity of measurement techniques and mathematical studies has made it challenging to establish standardized emission levels that are essential for comparative studies and a better understanding of the environmental impact of these emissions. Methane is considered a short-term climate amplifier since it is 28 times more likely to retain heat in the atmosphere than CO_2 or N_2O (Islam *et al.* 2022). Different publications point out that livestock farming is responsible for 14.5% of global greenhouse gases (GHG, Gerber *et al.* 2013), and dairy cows are liable for 18.9% of these emissions, mainly as CH_4 (Van Middelaar *et al*. 2014). In consonance with this and considering human consumption patterns and predicted global population growth, global enteric CH_4 emissions are estimated to increase by 70% by 2055 (Van Lingen *et al*. 2019).

In the current scenario, the prevailing perception is that progress in the dairy sector goes together with sustainable milk production, which is in line with preserving the environment by minimizing the carbon footprint, aligned with the Sustainable Development Goals (SDGs) adopted by the United Nations in 2015 (https://www.undp.org/).

When estimating greenhouse gases, there are many methods to choose from, each aimed at getting accurate and precise results for the emissions of each activity. These methods are meant to improve over time, moving towards more advanced approaches. The selection of the methodology falls into two types: 1) Methods based on observed emissions data; and 2) Methods based on calculation procedures that include emission factors (EF). Hence, there is an effort to develop empirical models in search of a comprehensive database that brings together data from various dairy production systems worldwide (Appuhamy *et al.* 2016; Jayasundara *et al.* 2016; Niu *et al.* 2018). This will enable the creation of reliable predictive models for use by on-farm veterinarians to balance ration composition with production, animal health, and the environment (Grainger and Beauchemin 2011). Nevertheless, no farmer is willing to favor strategies that negatively affect productivity, even if they effectively reduce CH_4 emissions (Martin *et al.* 2010).

Hence the importance international institutions such as The Intergovernmental Panel on Climate Change (IPPC, https://www.ipcc.ch) which has developed comprehensive guidelines for the creation of enteric CH_4 emission inventories from livestock, proposing different levels of calculation (also called Tiers) depending on the amount of information needed (including nutrition and production). Its predictive equations have been designed following the research on this issue in dairy cattle around the world. Tier 1 is an empirical method that uses default EFs per head of livestock to calculate enteric CH_4 emissions. Tier 2 is an enhanced method that

requires information about animal categories, feeding, and production systems. The Tier 3 approach is used only when a country-specific methodology for enteric CH_4 emission estimation has been developed by the IPCC. The Tier 2 method is recommended for countries with large livestock populations, using mostly national parameters related to feed diet, production characteristics, energy requirements, and energy/protein ratio, as is the case in Spain (Mshary *et al*., 2024).

One of the parameters to be determined with the Tier-2 approach is the calculation of methane emission factors (EF, kg CH_4/head/year) adapted to each production model, type of diet, and country, which contributes to the development of more detailed inventories. The result is the generation of friendly predictive equations for livestock that can be used on-farm. In this line, the National Greenhouse Gas Emissions Inventory Report by the Spanish Ministry for Ecological Transition and the Demographic Challenge (2022) encourages detailed studies in different Spanish regions and livestock conditions to provide accurate values.

The methods used to estimate enteric CH_4-EF (kg CH_4/head/year) included in the 2006 IPCC Guidelines and improved in 2019 (*The 2019 Refinement to the 2006 IPCC Guidelines, 2019*) provide an updated scientific basis to support the preparation and continuous improvement of national GHG inventories. The refinement consisted mainly of updating the CH_4 conversion factor (Ym %) and default EF (Cambra-López *et al*. 2008; Ibidhi *et al*. 2021). The parameter Ym (%) means the percentage of energy provided with the ratio converted to CH_4. This energy depends on several factors such as animal production and the quality and digestibility of the different diets. Additionally, considers milk production, the amount of neutral-detergent fiber (NDF) in the diet, as well as diet digestibility in their predictive formulae.

Considering the improvements that Galician dairy farms have experienced in the last decades and the urgent requirement to create greenhouse gas inventories on a national and global scale. This study aims to provide the first, currently non-existent, baseline data on CH_4 emission capacity in Galician dairy farms, and assess the role of milk yield, physiological state (peak lactation and dry-off period), and diet following the Tier-2 predictive model proposed by the IPCC. We point out that this study was conducted on commercial dairy farms under conditions different from those of a research center, whose results cannot always be transferred to field conditions.

1.2. MATERIAL AND METHODS

1.2.1. Selected farms, animals, and husbandry

We assessed twenty-one Holstein Frisian dairy farms that housed an average of 40 to 90 cows each. We selected these farms based on the 2019 Refinement to the 2006 IPCC Guidelines for high-producing dairy cattle (>8500 Kg/head/year) that receive diets with over 70% feed quality digestibility (DE%) according to the formula DE (MJ/kg)/GE (MJ/kg) x100 and less than 35% NDF (DMI basis). These high-producing cows have calved at least once and are used principally for milk production.

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Based on milk yield, we selected 9 farms with an average production between 38-40 Kgs of milk/cow/day, generating a mean annual output (at 305 days) within 11.590-12.200 Kgs of

milk/head/305-day lactation standard (Group-A; G-A). This cohort comprises a total of 630 multiparous dairy cows (700 ± 50 kg) with a mean dry matter intake (DMI) between 27.3 Kg/day in *peak lactation* (day 150 after calving) and 11.7 Kg/day in the *dry-off period* (60 days before calving).

We also select 12 farms with an average production ranging from 32-35 Kgs of milk/cow/day, generating a mean annual production (at 305 days) between 9.800-10.500 Kgs of milk/head/305-day lactation standard (Group-B; G-B). This cohort comprises a total of 900 multiparous dairy cows (700 ± 50 Kg) with a mean dry matter intake (DMI) of between 22.6 Kg/day during peak lactation and 10.5 Kg/day in the dry-off period. Overall, this study comprises 1530 dairy cows from two high-production groups.

The farms were selected considering similar management conditions to ensure applicable data for this type of exploitation and based on an intensive free-stall housing system and diet formulation. The diet after calving and during the lactation period consisted of a base ration, fed as a daily total mixed ration (TMR) comprising corn silage, grass silage, and a specific concentrate composed of corn, rapeseed meal, soybean flour, barley, and beetroot molasses. In the dry-off period, the diet was comprised mainly of corn silage, grass silage, and a commercial concentrate composed of rapeseed meal, barley, and beetroot molasses, according to National Research Council (NRC 2001) recommendations. **Table 5** displays the mean values of total concentrate and forage at each stage for both groups. Additionally, **Table 6** shows mean values of total dry matter intake (DMI) as feed, including kilograms of dry matter (and percentages) of forage and concentrate, for both groups of farms.

Table 5. Mean values of total dry matter intake (DMI) as feed, including kilograms of dry matter (and percentages) of forage and concentrate, for both groups of farms

Stage	Group	DMI (kg/d)
Peak lactation	**A** 11.590-12.200 Kgs milk/farm/ 305-day lactation standard	**Total: 27.3 kg.**
		Forage (%):15.7 (57.51 %)
		Concentrate (%):11.6 (42.49 %)
	B 9.800-10.500 Kgs milk/farm/305-day lactation standard	**Total: 22.6**
		Forage (%):11.7 (57.77 %)
		Concentrate (%):10.9 (48.23 %)
Dry-off	**A**	**Tota: 11,7**
		Forage (%):8.3 (70.94 %)
		Concentrate (%):3.4 (29.06 %)
	B	**Total: 10.5**
		Forage (%):7.7 (73.33 %)
		Concentrate (%):2.8(26.67 %)

Diet was fed *ad libitum* three times a day between 07:00-07:30, 14:00-14:30, and 20:00-20:30 h. TMR samples were collected at the beginning of each stage and sent for chemical analysis to the reference laboratory for all farms (Aira SCG, Lugo https://www.aira.es) using the NDS-Pro software platform (RUM&N Company, Italy RUM&N (https://www.rumen.it), developed in collaboration with the Cornell Department of Animal Science and based on the NRC (2001) standards.

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Milk production records were collected by the Friesian Breeders Association of Lugo (AFRICOR https://www.africorlugo.com). This legally authorized company recorded the daily yields of the animals previously identified with the farm code, bovine identification code, and calving date. In this way, it was possible to establish the total milk yields of the animals.

1.2.2. Development of Tier-2 Enteric Methane Emission Factors

Considering the estimations proposed by different studies (Appuhamy *et al.* 2016; Jayasundara *et al. 2016*; Niu *et al.* 2018) which have contributed to the IPPC Refinement (2019), we assume a Ym (%) value of 5.7 for lactating cows. For cows on each farm in the dry-off phase, the Ym (%) value will be 6.3. Later studies have also followed these assumptions (Niu et al. 2021).

The CH_4-EF for each dairy farm (G-A and G-B) were developed using the Tier-2 approach based on gross energy intake (GEI) and Ym (%) as follows: (Equation 10.21 from the 2019 Refinement to the 2006 IPCC Guidelines):

$$\underline{EF = [GE \times (Ym/100) \times 365 \text{ days})]}$$

$$55.65 \text{ MJ/kg } CH4$$

where EF is the CH_4 emission factor (kg CH4/head/year), and GE is gross energy intake (MJ/ head/day). The factor 55.65 (MJ/kg CH4) is the energy content of methane.

As a new aspect in this work and given that the IPPC itself recognizes a certain degree of uncertainty in its predictive equations, we wanted to provide, beyond general values, more precise data, based on the productive phases. Therefore, for each stage, we replaced the value 365 (overall value) with the corresponding number of days (150 for lactation peak and 60 for dry-off) seeking a reliable and precise calculation of the EF. This approach agrees with the criteria of Ricci et al. (2013) who consider that in addition to dietary characteristics, the physiological stage of the animal is necessary to improve the prediction of CH_4 emissions.

1.2.3. Statistics

Descriptive statistics were first computed for the key variables, including methane emission factors (kg CH_4/head/year), DMI (kg/day), and diet composition (e.g., crude protein or neutral detergent fiber). Means, standard deviations, and ranges were calculated to summarize the data and provide an initial understanding of the distribution of methane emissions and dietary intake across the two groups and physiological stages. Data normality and homogeneity of variances were checked using the Shapiro-Wilk and Levene's tests, respectively, before performing parametric tests.

To evaluate the differences in the chemical composition of diets between Groups A and B during peak lactation and the dry-off period, a series of statistical analyses were conducted. Independent t-tests were employed to compare the means of each dietary component for both groups at each stage. A significance level of $p < 0.05$ was established to determine statistically significant differences.

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To assess whether there were significant differences in methane emissions between G-A and G-B groups and for the physiological stages, we performed independent-sample t-tests and

paired t-tests where appropriate. Specifically, an independent-sample t-test was used to compare CH_4-EF between G-A and G-B groups during peak lactation and the dry-off period; the paired t-test was conducted to assess the differences in CH_4-EF within each group (G-A and G-B) between the peak lactation and dry-off periods. Given the exploratory nature of the study, a significance level of $P \leq 0.05$ was used for all tests.

On the other hand, multiple linear regression models were developed to explore the relationship between methane emissions (dependent variable) and independent variables such as DMI, diet composition and milk yield. The models were formulated as follows:

$$CH_4\text{-EF} = \beta_0 + \beta_1\ (\text{DMI}) + \beta_2\ (\text{CP}) + \beta_3\ (\text{EE}) + \beta_3\ (\text{NDF}) + \varepsilon$$

The goodness-of-fit for each model was assessed using the R-squared statistic, and the significance of each predictor was evaluated using P-values derived from t-tests on the regression coefficients.

Given the hierarchical structure of the data (cows nested within farms), mixed-effects models were employed to account for both fixed effects (e.g., diet composition, physiological stage) and random effects (e.g., variability between farms). This approach allowed for the assessment of within-farm and between-farm variability in methane emissions. The mixed-effects model was specified as:

$$CH_4\text{-EF} = \gamma_0 + \gamma_1\ (\text{Diet Composition}) + \gamma_2\ (\text{DMI}) + u_j + \varepsilon$$

These models are commonly utilized in animal nutrition and environmental science studies to analyze the impact of various dietary factors on greenhouse gas emissions (Sanchis et al. 2012)

Pearson correlation coefficients were calculated to determine the strength and direction of the relationships between pairs of variables, particularly focusing on CH_4-EF, DMI, and milk yield. Correlations were interpreted to understand how these factors interrelate and to identify potential multicollinearity in the regression models.

Finally, all statistical analyses were conducted using R (version 4.3.3), with packages such as lme4 for mixed-effects models, stats for regression and t-tests, and *ggplot2* for data visualization.

1.3. RESULTS

Significant differences were observed between Groups A and B during both peak lactation and the dry-off period (**Table 6**).

During peak lactation, Group A had higher dry matter (DM) (481 g/kg) and non-fiber carbohydrates (NFC) (280.7 g/kg) compared to Group B (388 g/kg DM and 270.8 g/kg NFC). Conversely, Group B exhibited higher crude fiber (CF) (180.6 g/kg) and acid detergent fiber (ADF) (218.6 g/kg) than Group A (172.8 g/kg CF and 209.6 g/kg ADF). Crude protein (CP) was also greater in Group B (166.8 g/kg) compared to Group A (157 g/kg). Gross energy intake (GEI) was significantly higher in Group A (486.01 MJ/kg DM).

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In the dry-off period, Group A maintained higher DM (685.3 g/kg) than Group B (582.5 g/kg), while CF levels were higher in Group B (228.1 g/kg vs. 253.2 g/kg). CP levels favored Group A (143.6 g/kg) over Group B (141.8 g/kg), but NFC was higher in Group B (141 g/kg). Starch levels were similar during peak lactation, but Group B had higher starch (81.3 g/kg) during the dry-off. Ether extract (EE) was consistently higher in Group B across both stages.

Table 6. Chemical composition (g/Kg DM basis) of cows in Groups A and B in peak lactation and dry-off stages. ***Different superscripts([a, b]) within the same physiological state group (peak lactation or dry-off) indicate significant differences at P ≤ 0.05***

	Peak lactation		*Dry-off period*	
Components[1]	**Group A**	**Group B**	**Group A**	**Group B**
DM (g/kg)	481[a]	388[b]	685.3[a]	582.5[b]
CF	172.8	180.6	253.2[a]	228.1[b]
aNDFmo	324.4	334.5	480[a]	461.6[b]
ADF	209.6	218.6	308.2[a]	290.6[b]
ADL	32.6	33.9	47.5[a]	41.4[b]
CP	157[a]	166.8[b]	143.6	141.8
NFC	280.7	270.8	131.4	141
Starch	232	231.9	66[a]	81.3[b]
EE	35.3[a]	38.3[b]	27.8[a]	30.2[b]
Ashes	76.8	76.8	91.4	92.3
GEI (MJ/kg DM)	486.01[a]	415.6[b]	143.6	181.9
UFL (MJ/kg DM)	43[a]	34.6[b]	52	43

[1]DMI: Dry matter intake, DM: Dry matter, CF: Crude fiber, aNDFmo: Neutral detergent fiber analyzed using a heat stable amylase and expressed inclusive of residual ash, ADF: Acid detergent fiber, ADL: Acid detergent lignin, CP: Crude protein, NFC: non-fiber carbohydrate, EE: Ether extract content; GEI: Gross energy intake.

The mean enteric methane emission factors (CH_4-EF) were higher in G-A compared to G-B across both physiological stages **(Figure 26).** Specifically, the mean CH_4-EF during *peak lactation* was 74.7 kg CH_4/head/year for G-A and 63.8 kg CH_4/head/year for G-B. During the dry-off period, the emissions significantly decreased to 14.0 kg CH_4/head/year in G-A and 12.3 kg CH_4/head/year in G-B.

The independent-sample t-test revealed a statistically significant difference in CH_4-EF between G-A and G-B during peak lactation ($P < 0.001$), with G-A producing 14.6% more enteric methane than G-B. Similarly, during the dry-off period, G-A cows emitted significantly more methane than G-B cows ($P = 0.014$), though the difference was smaller (12.1%). Paired t-tests indicated significant reductions ($P<0.001$) in CH_4-EF from peak lactation to the dry-off period within both groups, highlighting the impact of the physiological stage on methane emissions.

The multiple regression analysis identified DMI and EE content as significant predictors of CH_4 emissions. The final model explained a substantial proportion of the variance in CH_4-EF ($R^2 = 0.68$, $P < 0.001$). DMI was positively associated with CH_4-EF ($P<0.001$), indicating that higher intake increased methane production. In contrast, EE content was negatively associated with CH_4-EF ($P = 0.012$), suggesting that diets with higher fat content contributed to lower methane emissions.

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The random intercept model indicated significant variability between farms ($\sigma^2 = 3.2$, P = 0.021), the inclusion of farm-level random effects did not substantially alter the fixed effects estimates. This suggests that while there is some variability across farms, the observed effects of diet and DMI on CH_4-EF are consistent.

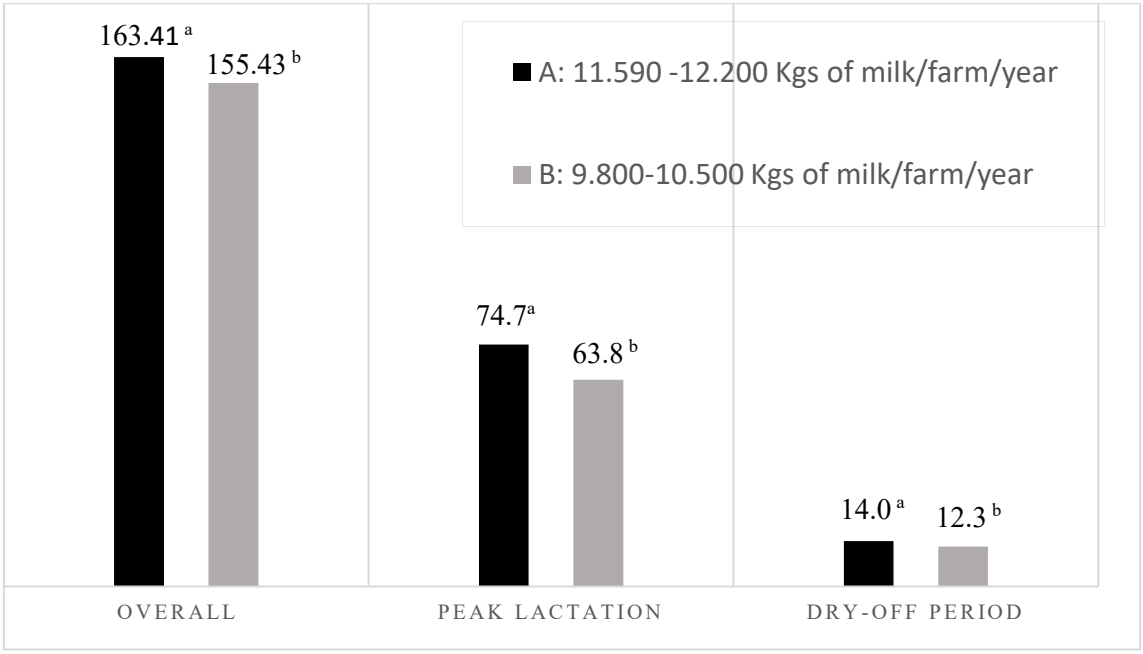

Figure 26. Variations in enteric methane emissions (EF, kg CH_4/head/year) values as a function of the physiological moment of the cows in the study. *Different superscripts(a, b) within the same physiological state group (overall, peak lactation or dry-off) indicate significant differences at P ≤ 0.05*.

Pearson correlation analysis revealed a strong positive correlation between CH_4-EF and DMI (R = 0.76, P < 0.001), and a moderate negative correlation between CH_4-EF and EE content (R = -0.45, P = 0.016). No correlation was found between CH_4-EF and CP or NDF, suggesting that these factors have no influence on methane emissions in this study.

Overall, our results indicate that enteric methane emissions are significantly influenced by both the physiological stage of lactation and diet composition, particularly DMI and EE content. The G-A group with higher milk production, emitted more methane than those in G-B, particularly during peak lactation.

1.4. DISCUSSION

1.4.1. Influence of Dry matter intake

The positive association between dry matter intake (DMI) and CH_4 emissions, as evidenced by both regression analysis and correlation results, aligns with existing literature indicating that increased feed intake leads to greater substrate availability for microbial fermentation in the rumen, thereby increasing methane production (Jaurena *et al.,* 2015; Van

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Gastelen *et al*., 2018; Hristov, 2024). In our study, DMI correlates with the physiological stage and its productive demands in high-yielding cows (Gross, 2022). This study shows that measurements of enteric methane in cows at the peak of lactation cannot be compared with measurements in the same cows at a less physiologically demanding time, supporting the notion that the physiological stage is a major determinant of CH_4 emissions (Ricci *et al.,* 2013; Hristov and Melgar, 2019).

When comparing the farm groups according to production stage, our findings are consistent with studies that suggest CH_4 emissions are higher in high-yielding dairy cows during peak lactation due to their greater feed intake and higher metabolic activity (Grainger and Beauchemin, 2011; Villanueva *et al*., 2023). Whereas in peak lactation methane production is related to lactose synthesis and milk fatty acids synthesis, these demands do not occur in the dry-off period (Van Gastelen *et al*., 2018). The higher CH_4-EF observed in G-A compared to G-B further corroborates this relationship, highlighting the importance of managing DMI and diet composition to mitigate emissions in high-producing cows (Hristov, 2024).

1.4.2. Impact of diet composition: The role of ether extract (EE) content

The negative relationship between EE (ether extract) content and methane emissions is particularly noteworthy. Diets higher in EE were associated with reduced methane emissions, a finding that supports the growing body of evidence suggesting that dietary fat can effectively suppress ruminal methanogenesis (Patra, 2013; Niu *et al.,* 2021). The potential mechanisms underlying this effect include the inhibition of methanogenic archaea and protozoa and alterations in ruminal fermentation pathways that favor propionate production over acetate and butyrate, which are precursors for methane (Cho *et al.,* 2024; Hristov 2024).

The higher EE content in the ration of G-B cows resulted in lower CH_4-EF values, suggesting that strategic dietary modifications could be a viable approach to mitigate methane emissions. However, the EE levels in the two rations used did not reach the threshold necessary to achieve the maximum potential in methane reduction (around 51 g/kg DM) described by Patra (2013). Further research can explore the effects of different fat sources and their impact on methane emissions to identify optimal dietary strategies (Bell *et al.,* 2016). Empirical evidence shows that GHG emissions per unit of animal product can be reduced by improving production efficiency and diet composition (Ouatahar *et al.,* 2021).

During peak lactation, Group A's higher dry matter (DM) and non-fiber carbohydrates (NFC) levels may contribute to enhanced energy intake and milk production (Hal *et al*., 2015). Increased NFC is often associated with higher methane emissions due to its fermentable nature, and more NDF will pass out of the rumen and be passed to the hindgut, where it can be fermented and produce CH_4 (Knapp *et al*., 2014). The elevated gross energy intake (GEI) observed in Group A could further exacerbate methane production, as higher energy diets can lead to increased fermentation activity in the rumen (Haque, 2018; Hristov *et al*., 2024). Conversely, while Group B exhibited higher crude fiber (CF) and acid detergent fiber (ADF) levels, these components are known to reduce methane emissions by promoting more efficient fermentation and increasing the passage rate of feed through the digestive system (Murray *et al*., 2019, Hristov 2024).

During the dry-off period, the dietary strategies of both groups continue to influence methane emissions. Group A's higher DM content may enhance nutrient absorption, but the transition to a higher fiber diet in Group B could lead to lower methane emissions (Van Gastelen

et al., 2018). The reversal in crude protein (CP) levels observed during this phase may also play a role, as protein fermentation in the rumen can lead to increased methane production (Knapp *et al*., 2014). The differences in starch content between the groups are particularly noteworthy. While both groups had similar starch levels during peak lactation, the higher starch content in Group B during the dry-off period could further mitigate methane emissions. Starch is less fermentable than NFC and can result in lower methane production when included in the diet (Hristov, 2024).

1.4.3. Influence of physiological stage

The overall EF values (163.41 and 155.43 kg CH_4/head/year for G-A and G-B farms, respectively) are higher than those recorded by the IPCC (2019) according to the Tier 2 model for dairy cattle in North America (EF = 138), Western Europe (EF = 126), Eastern Europe and Oceania (EF = 93), high productivity systems in Latin America (EF = 103), Asia (EF = 96), Africa (EF = 86), Middle East (EF = 94), or the Indian Subcontinent (EF = 70) (IPCC, 2019). The comparison of CH_4-EF for different types of farms is currently interfered with by significant discrepancies resulting from the diverse methodologies used in each published study (Niu *et al.,* 2018; Ibidhi *et al.,* 2021; Marumo *et al*., 2023). This situation complicates the comparison of these values with other systems worldwide and undermines efforts to contextualize them within our study.

In our view, and based on the formula developed by the IPCC, methane emission factors (CH_4-EF) have consistently been defined during the lactation phase. This definition involves calculating this stage's Gross Energy Intake (GEI) and multiplying it by 365 days. It is important to note that the observed phenomenon does not accurately represent the natural process, as cows go through a drying phase characterized by reduced milk production and changes in diet that influence the GEI. Therefore, it is essential to distinguish between the lactation and drying phases, recognizing that even during the dry-off period, methane emissions are still present despite the reduction in milk production. Considering these findings, we will compare the data obtained during the lactation phase with existing literature. If we restrict our investigation to the lactation phase, it becomes clear that despite significant differences, both groups exhibit lower values than the benchmarks previously mentioned for developed countries. This observation suggests that the assessment of CH_4-EF in dairy cows may be overestimated if it is not considered within a specific context.

In general terms, developed countries tend to emit more methane than developing countries due to their intensive farming practices (Sejian *et al*., 2011, Ricci *et al*., 2013). However, it is worth noting that grazing systems are not necessarily less polluting, as it is difficult to assess the chemical quality of grassland or even to measure individual methane emissions. This is an aspect where the prediction equations offered by the IPCC could be improved, as different farming systems are currently pooled together. It would be unfair to indicate that all intensive farms emit more methane than pasture-based farms, especially considering the multitude of studies that propose the mitigation of methane production through nutritional supplements (Ouatahar *et al.,* 2021; Lileikis *et al*., 2023).

When comparing the farm groups according to production stage, our findings are consistent with studies that suggest CH_4 emissions are higher in high-yielding dairy cows during peak lactation due to their greater feed intake and higher metabolic activity (Grainger and Beauchemin, 2011; Villanueva *et al*., 2023). Whereas in peak lactation methane production is related to lactose synthesis and milk fatty acids synthesis, these demands do not occur in the

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dry-off period (Van Gastelen *et al.,* 2018). The higher CH_4-EF observed in G-A compared to G-B further corroborates this relationship, highlighting the importance of managing DMI and diet composition to mitigate emissions in high-producing cows.

1.4.4. Variability between farms

The mixed-effects model revealed significant variability in methane emissions between farms, even after accounting for diet and DMI. This suggests that farm-specific factors, such as management practices, cow genetics, and environmental conditions, may also play a role in determining methane emissions (Sejian *et al.,* 2011, Ricci *et al*, 2013). Including random effects in the model underscores the importance of considering farm-level variability when developing mitigation strategies. Tailoring interventions to the specific conditions of each farm may enhance the effectiveness of methane reduction efforts. For this reason, the IPCC encourages field studies in cattle to be able to adjust the enteric methane emission values according to each condition.

1.4.5. Implications for mitigation strategies

The results of this study highlight the need for targeted mitigation strategies that consider both the physiological stage of the cows and the composition of their diet. While reducing DMI is not a feasible option given the production demands of high-yielding cows, adjusting the diet to include higher levels of EE and optimizing the balance of digestible fiber and non-structural carbohydrates could effectively reduce methane emissions without compromising milk yield. Additionally, the variability between farms suggests that personalized approaches, possibly involving precision feeding technologies, could further enhance the efficiency of methane mitigation strategies.

1.4.6. Limitations and future research

While this study provides valuable insights into the factors influencing methane emissions in dairy cows, several limitations should be acknowledged. First, the study was conducted under specific farm conditions in Galicia, and the results may not be directly generalizable to other regions or production systems. Second, the study primarily relied on predictive models (Tier-2) rather than direct measurements of methane emissions such as respiratory chambers. This approach allows for a more accurate and non-invasive determination of CH_4-EF on livestock farms, identifying favorable and detrimental factors on each farm and looking for nutritional options to reduce methane emissions without compromising farm productivity while addressing environmental concerns.

Additionally, conducting long-term studies to evaluate the sustainability and economic viability of dietary interventions designed to reduce methane emissions is crucial. Specifically unsaturated fatty acids in the ration decrease the populations of methanogens and protozoa in the rumen. As a result of these effects, the hydrogen (H_2) within the rumen increases, causing excessive amounts of reduced NADH to be channeled into propionate production. Furthermore, fat in the diet can inhibit butyrate production by suppressing microorganisms such as *Butyrivibrio fibrisolvens* in the rumen (Cho *et al.* 2024).

Under the conditions of our study, our data demonstrate that CH_4-EF in dairy cows is significantly influenced by dry matter intake, physiological stage, and diet composition, particularly the EE content. High-producing cows emit more methane, especially during peak

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lactation, but dietary strategies involving increased fat content could mitigate these emissions. The variability observed between farms suggests that individualized mitigation strategies could be particularly effective. These findings contribute to the growing body of knowledge needed to develop more sustainable dairy production systems that align with global greenhouse gas reduction targets developed by The Intergovernmental Panel on Climate Change (IPCC).

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CHAPTER 2.

Impact of soybean oil supplementation on enteric methane emissions in high-producing dairy cows using a Tier-2 model

2.1. INTRODUCTION

The study of methanogenesis in ruminants has been for decades an area of great interest in veterinary medicine for two reasons: the energy consumption involved in the production of methane at the expense of other body functions (growth, reproduction, lactation, etc.) and the presence of diseases associated with the excessive generation of this gas.

For just over a decade, methane and ruminants have been linked again, but now from an environmental point of view, as cattle are considered to be one of the most important anthropogenic greenhouse gas (GHG) producers, with methane standing out for its properties, considered to be a short-term climate intensifier, as its potential to trap heat in the atmosphere is 28 times greater than that emitted by CO_2 or N_2O (Islam *et al*., 2022). According to recent publications, the livestock sector is responsible for 14.5% of the global GHG (Gerber *et al*., 2013) and dairy cattle account for 18.9% of these emissions, mainly in the form of enteric CH_4 emissions (van Middelaar *et al*., 2014).

Conscious of a reality that is increasingly being implemented worldwide, it is time for veterinary research and its acquired experience to be part of the solution and not of the problem using adequate nutritional management. In this way, we can help the farmer to keep profitability on the farm. According to Martin *et al.,* (2010), no livestock producer would favor strategies that negatively affect productivity, even if they effectively reduce CH_4 emissions of ruminants. For this reason, the most effective approach is prevention, which involves a detailed study of the physicochemical components of the ration and their interaction in the rumen.

Hence the importance of international institutions such as The Intergovernmental Panel on Climate Change (IPPC) which has developed comprehensive guidelines for the creation of enteric CH_4 emission inventories from livestock, proposing different levels of calculation (also called Tier) depending on the amount of information needed (including nutrition and production). Its predictive equations have been designed following the research carried out on this issue in dairy cattle around the world, with their peculiarities. However, to the best of our knowledge, to date, there are no studies about the dairy cattle system in Spain.

One of the parameters to be determined is the calculation of methane emission factors (EF, kg CH_4/head/year) adapted to each production model, type of diet, and country, which contributes to the development of more detailed inventories. The result is the generation of nutritionist-friendly predictive enteric methane emission equations for livestock that can be used on-farm to balance ration composition with production, animal health, and the environment.

Under this scenario, within a research project that is being conducted on methane emission profiles in dairy farms in Galicia (Spain), we found a farm that maintaining its high milk production showed lower rates of CH_4-EF than other nearby high-yielding dairy farms,

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hypothetically attributable to the diet received since all dairy farms had the same breed and were under the same management conditions.

Therefore, the objective of this work is to evaluate, at on-farm conditions, which physicochemical components of the ration could have contributed to these lower emissions, since it is feasible that a reformulation of the rations, according to production and physiological state of animals, may be part of the solution for this problem.

2.2. MATERIAL AND METHODS

2.2.1. Animals, diets and experimental design

Ten dairy farms were selected taking into consideration the *2019 Refinement to the 2006 IPCC Guidelines* for high-producing dairy cattle (>8500 Kg/head/year) that receive diets with over 70% digestible energy (DE%), expressed as a percentage of Gross Energy (GE) ratio, according to the formula DE (MJ/kg)/GE (MJ/kg) x100 and less than 35% neutral detergent fiber (NDF, DMI basis). These farms have an average production between 38-40 Kg of milk/cow/day, generating a mean annual output (at 305 days) within 11.590-12.200 Kg of milk/farm/305-day lactation standard. This cohort comprises a total of 720 multiparous dairy cows (700 ± 50 kg) with an estimated mean dry matter intake (DMI) between 27.3 Kg/day in peak lactation (day 150 after calving) and 11.7 Kg/day in the dry-off period (60 days before calving).

Milk production records were collected monthly by the Friesian Breeders Association of Lugo (AFRICOR https://www.africorlugo.com). This legally authorized company recorded the daily yields of the animals previously identified with the farm code, bovine identification code, and calving date.

The farms were selected based on the criterion that all cows were housed in a free-stall housing system with a similar diet and with the farmer's approval. After selecting and analyzing the farms, we found that all farms except one were using monounsaturated fatty acid (MUFA) calcium soaps as a lipid supplement, while the other farm was using soybean oil. We then investigated the potential effects of these different lipid supplements on CH_4-EF values and divided the farms into two groups: F-X farm (n= 90 cows supplemented with soybean oil, 0,32% DM)) and the remaining nine farms, F-9 farms, which were supplemented with calcium soaps (0,73% DM, n=630 cows).

The diet after calving and during the lactation period consisted of a base ration, fed as a daily total mixed ration (TMR) comprising corn silage, grass silage, and a specific concentrate composed of corn, rapeseed meal, soybean flour, barley, and beetroot molasses. During this phase, both groups were given lipid supplementation (soybean oil for F-X and calcium soaps, F-9). In the dry-off period, the diet consisted of corn silage, grass silage, and a commercial concentrate made of rapeseed meal, barley, and beetroot molasses, according to NRC (2001) recommendations.

The estimated average dry matter intake for each group (DMI) is reflected in **Table 7**. This estimation was done using the Cornell Net Carbohydrate and Protein System (CNCPS), a mathematical model that estimates cattle requirements and nutrient supply based on feed compositional information in diverse production situations.

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Table 7. Mean values of total dry matter intake (DMI) as feed, including kilograms of dry matter of forage and concentrate, for both groups of farms.

Stage	Group	DMI (kg/d)	DMI (kg)
Peak lactation	**F-X**		**22.8**
		Total forage	10.6
		Total concentrate	12.2
	F-9		**27.3**
		Total forage	15.7
		Total concentrate	11.6
Dry-off	**F-X**		**11.0**
		Total forage	7.9
		Total concentrate	3.1
	F-9		**11.7**
		Total forage	8.3
		Total concentrate	3.4

The diet was fed ad libitum three times per day between intervals 07:00-07:30, 14:00-14:30, and 20:00-20:30 h. TMR samples were collected and sent for chemical analysis to the reference laboratory of all farms (Aira SCG, Lugo https://www.aira.es) using the NDS-Pro software platform (RUM&N Company, Italy RUM&N (https://www.rumen.it), developed in collaboration with the Department of Animal Science at Cornell. The chemical composition of the diets during the lactation and drying periods is shown in **Table 8**

Table 9 reflects the consumption of the amount of different fatty acids (FA) of the TMR in the farms tested.

Table 8. Chemical composition (g/Kg DM basis) of cows for both groups in peak lactation and dry-off stages.

	Peak lactation			*Dry-off period*		
Item[1]	**F-X**	**F-9**	**SD**	**F-X**	**F-9**	**SD**
DMI (Kg/head/day)	22.8	27.3	1.24	11.7	11.7	0.43
DM (g/kg)	522	481	21.6	632	685.3	**70.5**
CF	163.7	172.8	9.42	208.3	253.2	21.6
aNDFmo	300.3	324.4	4.71	447.4	480	28.67
ADF	191.6	209.6	4.32	278	308.2	26.24
ADL	30.9	32.6	0.29	41	47.5	0.92

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CP	166.3	157	4.74	161.4	143.6	4.63
NFC	321	280.7	16.9	136.8	131.4	8.16
Starch	239.1	232	12.4	57.1	66	9.40
EE	37.3	35.3	3.47	32.5	27.8	4.17
Ashes	71.4	76.8	4.9	93.8	91.4	8.16
GEI diet (MJ/kg DM)	12.8	18.6	0.7	13.8	12.3	0.30
GEI (MJ/kg DM)	292.2	486.0	20.5	152.8	205	5.71

[1]DMI: Dry matter intake, DM: Dry matter, CF: Crude fiber, aNDFmo: Neutral detergent fiber analyzed using a heat stable amylase and expressed inclusive of residual ash, ADF: Acid detergent fiber, ADL: Acid detergent lignin, CP: Crude protein, NFC: non-fiber carbohydrate, EE: Ether extract content; GEI diet (MJ/kg DM): Gross energy content. GEI: Gross energy intake.SD: Standard deviation.

Table 9. Fatty acid composition of the TMR (g/Kg DM basis) in both groups at peak lactation

Group[1]	Name	F-X	Mean F-9
SFA	C12:0 (Lauric acid)	9.7	0.33
SFA	C14:0 (Myristic acid)	6.4	6.7
SFA	C16:0 (Palmitic acid)	89.4	117.8
SFA	C18:0 (Stearic acid)	15.2	13.0
MUFA	C16:1 (Palmitoleic acid)	3.4	0.41
MUFA	C18:1 (Oleic acid) (*cis* + *trans*)	134.2	139.3
PUFA	C18:2 (Linoleic acid)	268.6	142.2
PUFA	C18:3 ALA (α-linoleic acid)	85.4	11.9
LCFA	Long chain fatty acids	24.01	8.8

[1]SFA: Saturated fatty acid; MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid

2.2.2. Development of Tier 2 Enteric Methane Emission Factors

The 2019 Refinement to the 2006 IPCC Guidelines, establishes that "in the absence of country or region-specific methane emission factor (Ym %) values the calculation of CH_4-EF should be done assuming values of Ym (%) based on production, diet digestibility, and NDF content".

The refinement consisted mainly of an update of the CH_4 conversion factor (Ym%) and the default EF. The parameter Ym (%) means the percentage of energy provided with the ration converted into CH_4 (IPCC, 2019) This energy depends on several factors, such as animal production and the quality and digestibility of the different diets.

Considering the estimations proposed by different studies (Fox et al., 2004; Appuhamy et al., 2016; Niu et al., 2018) which have contributed to above mentioned IPCC Guidelines we assumed a Ym (%) value of 5.7 for lactating cows. For cows on each farm in the dry-off period, the Ym (%) value was established at 6.3. Later studies have also followed these assumptions (Jayasundara et al., 2016; Niu et al., 2021). The CH_4-EF for each dairy farm was calculated using the IPCC Tier 2 approach based on gross energy intake (GEI) and Ym (%) (represents the proportion of the GEI that is converted into methane during the fermentation process in the rumen) as follows: (Equation 10.21 from the 2019 Refinement to the 2006 IPCC Guidelines):

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$$\underline{EF = [GE \times (Ym/100) \times 365 \text{ days})]}$$

$$55.65 \text{ MJ/kg } CH4$$

where EF is the CH_4 emission factor (kg CH_4/head/year), and GE is gross energy intake (MJ/ head/day. The factor 55.65 (MJ/kg CH4) is the energy content of methane. For each stage, we replaced the value 365 (overall value) with the corresponding number of days (150 for lactation peak and 60 for dry-off) seeking a reliable and precise calculation of the EF in each phase according to DMI and production. This approach agrees with the criteria of Ricci *et al*. (2013) who consider that in addition to dietary characteristics, the physiological stage of the animal is necessary to improve the prediction of CH_4 emissions.

2.2.3. Statistics

The study involved a cohort of 720 multiparous dairy cows from ten high-production dairy farms. Nine of these farms (called F-9) used a diet supplemented with calcium soap salts, while one farm (called F-X) used soybean oil as the dietary supplement. The unbalanced nature of this study design arises from the disparity in sample sizes between the groups: 630 cows in the F-9 farms and 90 cows in the F-X farm.

Variables were categorized as follows: The independent variable was the type of lipid supplementation (soybean oil vs. calcium soap salts); the dependent variable was enteric methane emissions, expressed as emission factors (EF, kg CH_4/head/year); and the covariates were dry matter intake (DMI), fatty acid composition, and physiological stages (peak lactation and dry-off).

Given the unbalanced nature of the design, a mixed-effects linear model was employed to account for the variability within and between farms. The model included the type of lipid supplementation as a fixed effect and farm as a random effect to control for farm-specific characteristics. Differences in methane emissions between the two groups were assessed using pairwise comparisons with a significance level set at $p < 0.05$.

For the analysis of variance in methane emissions, an ANOVA was applied, followed by post hoc tests to compare the emission levels between the groups, adjusted for multiple comparisons using the Bonferroni correction. Additionally, regression analysis was performed to explore the relationship between fatty acid composition in the diet and methane emissions.

2.3. RESULTS

Results (**Figure 27**) showed a significant difference in methane emissions between cows on the F-X farm, supplemented with soybean oil, and cows on the F-9 farm, supplemented with calcium soap salts ($p < 0,05$). The type of diet had a significant impact on methane emissions. The soybean oil diet led to a significant decrease in methane emissions compared to the calcium soap salts diet, with the model accounting for 38% of the variance in emissions. The F-X farm, which used soybean oil supplementation, had a lower mean enteric me-thane emission factor (EF = 109.25 kg CH_4/head/year) compared to the F-9 farms (EF = 163.41 kg CH_4/head/year). The DMI and fatty acid composition varied between the two groups, with F-X cows consuming less forage but similar amounts of concentrate compared to F-9 cows.

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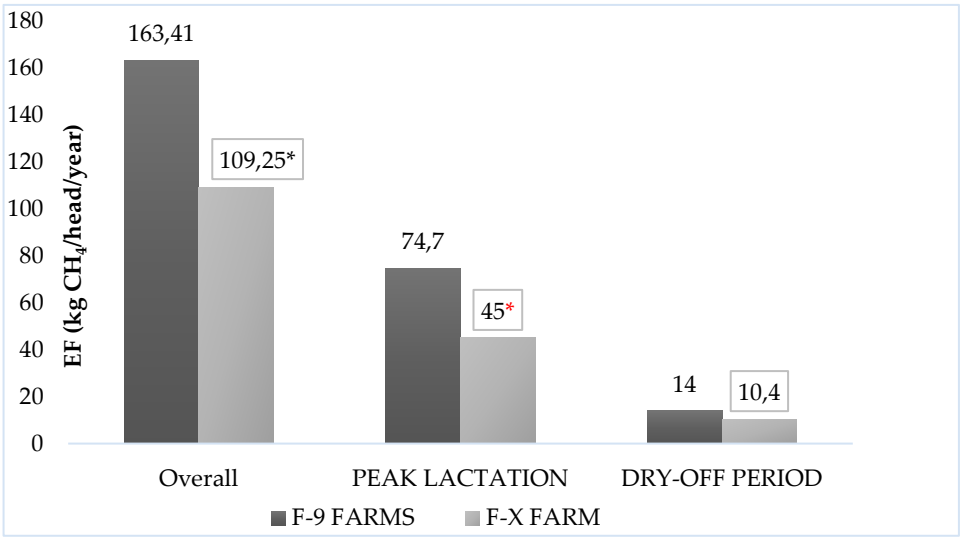

Figure 27. Tier 2 EF (kg CH_4/head/year), and the changes observed according to physiological status (peak lactation and dry-off) for each studied group.

The ANOVA analysis confirmed significant differences in methane emissions between the groups ($F(1, 9) = 12.34$, $p < 0.01$). Post hoc tests showed that the F-X farm's emissions were significantly lower than those of any individual F-9 farm, even after adjusting for multiple comparisons. Regression analysis demonstrated a significant negative correlation between the intake of certain fatty acids (e.g., C12:0, C18:2, C18:3 ALA) and methane emissions ($R^2 = 0.65$, $p < 0.05$), suggesting that these fatty acids contribute to the reduction in methane production observed in the F-X group.

2.4. DISCUSSION

Seijan *et al*. (2011) pointed out that developed countries tend to emit more me-thane than developing countries due to their intensive farming practices. However, the results found in the F-X farm do not agree with their conclusions, since these cows were reared under the same conditions as the cows of the other nine farms. These exceptionalities are the reason why the IPCC encourages the development of studies on enteric me-thane emissions in ruminants under different farm conditions worldwide, where parameters such as management, diet, etc. come into play. The current values given by IPCC (2019) are very generalized because of the pooling of different management systems. Pointing out that in a developed country like Spain, there may be farms emitting methane below the average provided by other similar countries may help to establish more accurate predictive values. However, it is necessary to examine the factors that have led to this unexpected difference compared to the other nine farms in the study.

The metabolic status characteristic of lactation, linked to feed intake and milk yield is associated with higher enteric methane emissions in comparison with other productive phases, as previously described by Mahdavi *et al*. (2019) or Villanueva *et al*. (2023) in Holstein dairy cows, with the highest emissions occurring at the peak of lactation. To accurately assess methane emissions in cattle, it is important to not just look at overall values but to provide data

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specific to relevant production phases. In dairy cows, this means focusing on phases such as lactation and dry-off for dairy cattle, as recommended by Ricci *et al.* (2013).

The lower CH_4-EF values for F-X in peak lactation in comparison with F-9 can be attributable to the composition of the feedstock. The F-X farm consumed less forage, and a similar amount of concentrate compared to F-9, suggesting that the quality of the silage administered might have played a determining effect based on its composition (Hegarty *et al*., 2021).

In our case, the high forage intake relative to concentrates, along with the higher DM content on F-X farms, results in an increased fiber content, which ferments in the rumen and promotes hydrogen (H_2) synthesis. This hydrogen is subsequently utilized by methanogens to produce methane (Ramin and Huhtanen, 2013; Beauchemin *et al*., 2022). Conversely, the lower forage-to-concentrate ratio consumed by cows on the F-X farm increases the risk of rumen pathology due to a decrease in pH (Roques *et al*., 2024). However, this shift also directs rumen fermentation towards propionate production, which acts as an electron sink and reduces the availability of hydrogen for methanogens, thereby decreasing methane production (Beauchemin *et al.,* 2022; Tseten *et al*., 2022). Increased DMI generally leads to higher methane emissions because more organic matter is available in the rumen for fermentation, producing methane as a byproduct (Martin *et al*., 2010; Beauchemin *et al*., 2022; Roques *et al*., 2024).

Unfortunately, the physicochemical characteristics of forage were not determined for our current study because it was not the initial objective of the research. Although the TMR analysis shows a higher amount of fat (EE) in F-X, it also reflects a lower contribution of non-digestible fiber (ADF) and aNDFom. In addition, the higher contribution of C12:0; C16:1; C18:2; C18:3 ALA (α-linoleic acid) and long chain fatty acids (LCFA) and lower C16:0 in the F-X group compared to the other nine farms (F-9) is notable (Maia et al., 2007; Maigaard et al., 2024).

This finding suggests that nutritionists need to know which FA combinations can optimize cows' productive responses at different physiological phases and their impact on CH_4-EF emissions (Maia et al., 2007). In addition, considering the volume of forage consumed by the cows in this study, it would be helpful to include the FA composition of the forage in further studies. This information rarely appears in field trials or nutritional studies.

Numerous studies address the effect of different FAs on the digestibility of different nutrients and their effect on milk production and methane emissions. And our study is no exception. Dietary fats have a moderate potential for CH_4 reduction and have been evaluated in different reviews and meta-analyses [Beauchemin *et al*., 2007; Martin *et al*., 2020)].

Lipids replace fermentable organic matter (OM) in diets with nonfermentable OM, resulting in less available substrate (H_2) for methanogenesis (Patra, 2013; Beauchemin *et al*., 2022). This is quantitatively the most important effect of fat for CH_4 reduction (Roques *et al*., 2024). Furthermore, the inclusion of fatty acids, depending on chain length and degree of saturation, inhibits the populations of protozoa and methanogens in the rumen, and biohydrogenation of unsaturated fatty acids in the rumen redirects H_2 away from methanogenesis (Alstrup *et al*., 2015) enhancing N-digestibility (Beauchemin *et al.,* 2007) being able to obtain the maximum benefit from the TMR. Patra (2013) reported that C12:0 and C18:3 fatty acids have the strongest CH_4 suppressive effect. On the other hand, LCFA is an energy substrate that contributes to decreasing methanogenesis by reducing rumen acetate utilization, as demonstrated by (Ramin, and Huhtanen, 2013; Hua *et al*., 2022) in lactating cows.

The *in vitro* studies of Betancur-Murillo *et al.* (2023) demonstrated that soybean oil increases the population of *Prevotella ruminicola* which can divert the flow of hydrogen in glycolysis away from methanogenesis favoring the production of propionic acid. Furthermore, it plays an active role in lignocellulose degradation (ADF and ADL) associated with reduced methane emissions (Ramin and Huhtanen, 2013; Mahdavi *et al*., 2019).

Therefore, the addition of soybean oil has a beneficial effect on the rumen degradation of the ration administered in lactation according to its properties, being able to obtain the maximum benefit from the ration provided (Tseten *et al*., 2022). The potential for feeding soybean oil on commercial farms as a methane mitigation strategy is high because it represents a "natural" intervention rather than processed fat supplements. The challenge lies in its economic cost.

In the dry-off period, when no lipid supplements were added, cows on the F-X farm fed less aNDFmo, ADF, ADL, and starch (Hegarty *et al*., 2021). And yet, it produced % less enteric methane emissions. Considering that the DMI for dry cows is almost the same in both groups: 11.0 and 11.7 kg DM (only 6% higher in F-9 farms), the decrease in methane emissions can be attributed to the lower intake (Ramin and Huhtanen, 2013; Roques *et al*., 2024).

The differences concerning this item can be attributable to supplementation with soybean oil versus other fat supplements in the TMR. The potential for applying lipid feed such as soybean oil as a methane mitigation strategy is high because these sources represent "natural" lipid supplements, more appreciated environmentally and by consumers.

While we recognize that the weakness of this study is the lack of the fatty acid profile of the supplied forage, we have found that the type of lipid supplementation determines the greater or lesser methane emission in dairy cattle.

This implies that veterinarians, based on the data provided through Tier-2 predictions, can adapt the rations of animals at different productive moments with the dual purpose of minimizing greenhouse gas emissions and not threatening the profitability of the farm.

This study demonstrates the significant impact of dietary lipid supplementation on enteric methane emissions in high-yielding dairy cows. Specifically, the inclusion of soybean oil in the diet significantly reduced methane emissions compared to calcium soap salts. The results suggest that the fatty acid composition of the diet, particularly the presence of specific fatty acids such as C12:0, C16:1, C18:2, C18:3 (ALA) (Maia *et al*., 2007; Maigaard *et al*., 2024; Roques *et al*., 2024) and long-chain fatty acids, plays a critical role in influencing methane production. These findings highlight the importance of considering diet composition in strategies aimed at mitigating greenhouse gas emissions from dairy farming.

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CHAPTER 3.

Effect of lipid supplementation on enteric methane emissions and on the physico-chemical quality of milk in high yield dairy cows.

3.1. INTRODUCTION

Methane production in cows is closely related to their diet. It is well known that cows consuming high forage diets (such as grass and hay) tend to produce more methane, due to a higher production of H_2, which is used by methanogenic *archaea*. Conversely, increasing the amount of concentrates (such as grains and cereal by-products) reduces this production. However, this alternative is debatable because apart from competing with human feed, it has been shown to cause numerous ruminal disturbances, such as ruminal lactic acidosis.

For this reason, the most effective approach is prevention, which involves a detailed study of the physicochemical components of the ration and their interaction in the rumen. Among various methane mitigation technologies documented, the *dietary strategy* appears to be one of the promising technologies to curb methane emissions from ruminants while improving the productivity of animals (Martin *et al*., 2010; Patra, 2012).

Supplementation of fatty acids (FA) to the feed decreases CH_4 production in the short term (Martin et al., 2008; Brask et al., 2013) and is one of the most promising dietary strategies to reduce enteric CH_4 production (Hristov et al., 2013). However, the persistence of this reduction has been questioned (Woodward et al., 2006; Hristov et al., 2013). Fat supplementation reduces the fermentable substrate and can lower organic matter (OM) and fibre degradability (Knapp et al., 2014). Consequently, the amount of H_2 produced during fermentation is reduced, resulting in decreased CH_4 production.

Besides, supplementation with fat may reduce the activity of ruminal methanogens and protozoal numbers (Hristov et al., 2013). An increased degree of FA unsaturation will increase the negative effect on fibrolytic bacteria and methanogens (Giger-Reverdin et al., 2003). Supplementation with fat increases energy density in the diet and often increases milk yield (Larsen et al., 2012). However, the response in milk yield to supplementary fat is complex and not easy to predict (Garnsworthy, 1997). The supplementation of fat often replaces easily digestible carbohydrates thereby reducing fermentation and changing the fermentation pattern in the rumen.

Calcium soaps are used in dairy nutrition to enhance the energy density of the diet without negatively affecting rumen fermentation, mainly composed of palm oil. They are designed to bypass the rumen and be digested in the intestines. This helps in preventing the negative effects of unsaturated fats on rumen fermentation, thus maintaining optimal rumen function. Additionally improves energy intake increasing the energy density of the diet. This is

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particularly beneficial for high-producing dairy cows that have high energy demands, and enhance milk yield and composition (Varga-Bello, 2020).

There are few studies on the effects of long-term feeding strategies to reduce CH_4 production, as the rumen microflora seems to adapt to different CH4 inhibitors (Johnson and Johnson, 1995). Woodward et al. (2006) suggest that this may also apply to fat supplementation and the type of fat, as some may be synthetically produced (calcium soaps) or derived from plant extracts or oils.

The present study aimed to investigate the long-term effect of fat supplementation in two different forms (palm oil calcium soap and soya oil) on CH_4 production in dairy cows and the physicochemical characteristics of the milk produced.

3.2. MATERIAL AND METHODS

3.2.1. Selected farms, animals, and husbandry

Four Holstein Frisian dairy farms located in Galicia (N.W Spain) were evaluated, with an average of 75 to 90 cows on each farm. The farms were carefully selected based on the *2019 Refinement to the 2006 IPCC Guidelines for high-producing dairy cattle* (>8500 kg/head/year) that receive diets with over 70% feed quality digestibility (DE%) according to the formula DE (MJ/kg)/GE (MJ/kg) x100 and less than 35% NDF (DMI basis). These high-producing cows have calved at least once and are primarily utilized for milk production.

Based on milk yield, we selected 4 farms with an average production between 38-40 Kgs of milk/cow/day, generating a mean annual output (at 305 days) within 11.590-12.200 Kgs of milk/head/305-day lactation standard. This cohort comprises a total of 335 multiparous dairy cows (700 ± 50 kg) with a mean dry matter intake (DMI) between 27.3 Kg/day in peak lactation (day 150 after calving).

In addition, we selected the farms to be representative of the standard diets (in the form of TMR rations) fed to dairy cattle farms in the study area (**Table 10**). The only difference we looked for was the type of fat supplementation given to the ration: calcium salt soaps (palm oil based, n=3) or soybean oil (n=1). In this way, the farms were classified into 4 batches depending on the type and quantity of lipid supplementation: Group **70-SO** (n= 90 cows), supplemented with 70 gr. of soybean oil (participation in the Ethereal Extract (EE) of the ration of 8.5%); Group **150 CS** (n=75 cows) supplemented with 150 gr. of calcium soap based on palm oil (12.7% of the total EE); Group **210-CS** (n= 80 cows) supplemented with 210 gr (12.7% of the total EE) and finally Group **250-CS** (n= 90 cows) which was supplemented with 250 gr. of the same type of calcium soap (19.9% of the total EE content of the ration).

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Table 10. Chemical composition (g/Kg DM basis) of cows in the different studied groups

Group	DM[1] (g/kg/DM as feed)	aFNDmo (%)	ADF (%)	ADL (%)	NFC (%)	Starch (%)	CP (%)	EE (%)	Ash (%)
70-SO	522	300.3	191.6	30.9	320.9	239.1	166.3	37.3	71.4
150-CS	452	322.1	208.8	33.3	298.8	247.5	161.0	33.2	76.8
210-CS	493	329.6	213.0	35.2	266.6	216.7	149.7	36.8	83.9
250-JCS	498	321.4	207.1	29.4	276.8	231.4	160.1	35.9	69.6

[1]DM: Dry matter, aNDFmo: Neutral detergent fiber analyzed using a heat stable amylase and expressed inclusive of residal ash, ADF: Acid detergent fiber, ADL: Acid detergent lignin, NFC: non-fiber carbohydrate, CP: Crude protein, EE: Ether extract content; GEI: Gross energy intake.

The gross energy intake (MJ/day) for each group was as follows: 292.24 (70-SO), 458.04 (150-CS); GEI (MJ/day); 508 (210-CS) and 492 (250-CS). Diet was fed *ad libitum* three times a day between 07:00-07:30, 14:00-14:30, and 20:00-20:30 h. TMR samples were collected at the beginning of each stage and sent for chemical analysis to the reference laboratory for all farms (Aira SCG, Lugo https://www.aira.es) using the NDS-Pro software platform (RUM&N Company, Italy RUM&N (https://www.rumen.it), developed in collaboration with the Cornell Department of Animal Science and based on the NRC (2001) standards.

Milk production records were collected by the Friesian Breeders Association of Lugo (AFRICOR https://www.africorlugo.com). This legally authorized company recorded the daily yields of the animals previously identified with the farm code, bovine identification code, and calving date. In this way, it was possible to establish the total milk yields of the animals. Milk parameters used for this study were in (%): fat, protein, beta-hydroxybutyrate, monounsaturated fatty acids (MUFA and polyunsaturated fatty acids (PUFA)

3.2.2. Statistics

Levene's test was used to ensure that the data in each group followed a normal distribution, which evaluates the null hypothesis that the population variances are equal. When assessing the equality of variances between the groups, we found a P-value > 0.05, indicating no significant differences between the variances of the groups.

The *Independent Samples t-test* was used to determine if there were differences between the means of the two independent groups. The t-value was calculated using the means, standard deviations, and sample sizes of the two groups, as well as the P-value to determine the significance of the results. A significant difference is considered when the p-value is > 0.05.

An independent samples t-test requires some assumptions: 1) independent observations; 2) normality: the dependent variable must follow a normal distribution in each subpopulation and 3) homogeneity of variances: both subpopulations must have equal variances on the dependent variable.

The following formula was used for the calculation of the *t-statistic* and to determine whether there is a significant difference between the means of two groups, taking into account degrees of freedom:

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t-statistic: [t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}] where:

- (\bar{X}_1) and (\bar{X}_2): Sample means of the two groups
- (s_1^2) and (s_2^2): Sample variances of the two groups
- (n_1) and (n_2): Sample sizes of the two groups

Degrees of Freedom (df): [df = \frac{\left(\frac{s_1^2}{n_1} + \frac{s_22}{n_2}\right)2}{\frac{\left(\frac{s_12}{n_1}\right)2}{n_1 - 1} + \frac{\left(\frac{s_22}{n_2}\right)2}{n_2 - 1}}]

For all these determinations, we used the statistical program Python, utilizing SciPy's library for Levene's test and the t-test; NumPy's library for data analysis and visualization and Panda's library to calculate correlations and Analysis of Variance (ANOVA) in the case of milk components.

To calculate the correlation between enteric methane emissions and physicochemical values of milk we use the following correlation matrix:

[CH_4-EF & FAT & PROTEIN & BHB & MUFA & PUFA \hline \CH_4-EF & 1.000 & -0.000 & -0.107 & 0.371 & -0.253 & -0.517 \ FAT & -0.000 & 1.000 & -0.966 & 0.364 & 0.967 & 0.856 \ PROTEIN & -0.107 & -0.966 & 1.000 & -0.591 & -0.916 & -0.778 \ \BHB & 0.371 & 0.364 & -0.591 & 1.000 & 0.288 & 0.141 \ MUFA & -0.253 & 0.967 & -0.916 & 0.288 & 1.000 & 0.959 \ PUFA & -0.517 & 0.856 & -0.778 & 0.141 & 0.959 & 1.000

Considering the estimations proposed by different studies (Appuhamy et al. 2016; Jayasundara *et al.,* 2016; Niu *et al*., 2018) which have contributed to the IPPC Refinement (2019), we assume a Ym (%) value of 5.7 for lactating cows. For cows on each farm in the dry-off phase, the Ym (%) value will be 6.3. Later studies have also followed these assumptions (Niu *et al.,* 2021).

The CH_4-EF for each dairy farm (G-A and G-B) were developed using the Tier-2 approach based on gross energy intake (GEI) and Ym (%) as follows: (Equation 10.21 from the 2019 Refinement to the 2006 IPCC Guidelines):

$$\underline{EF = [GE \times (Ym/100) \times 365 \text{ days})]}$$

$$55.65 \text{ MJ/kg } CH4$$

where EF is the CH_4 emission factor (kg CH4/head/305 DIM), and GE is gross energy intake (MJ/ head/day). The factor 55.65 (MJ/kg CH_4) is the energy content of methane.

3.3. RESULTS

When comparing the physico-chemical values of the four rations, no differences were found (P>0.05) according to the type of supplementation, except for the energy provided by the ration (GEI), where the lowest energy intake was found for the ration supplemented with soybean oil.

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The descriptive analysis (**Figure 28)** shows considerable variability (P<0.001, t=-14.14) in CH_4-EF values, with the lowest emissions for 70-SO and the highest in 210-CS. When comparing emissions between the 3 groups receiving CS there are no differences in enteric emissions (P= 0.26).

This result suggests that the factors influencing the energy of the ration also affect enteric methane emissions. Specifically, increasing energy through lipid supplements such as calcium soaps implies a higher production of enteric methane, regardless of the dose. Since the EE content of the ration did not change based on the type of supplement (soybean oil or palm calcium salts), this suggests that is the type of the fat supplement that determines the variations in greenhouse gas emissions (GEI) as well as in methane emission factor (CH_4-EF).

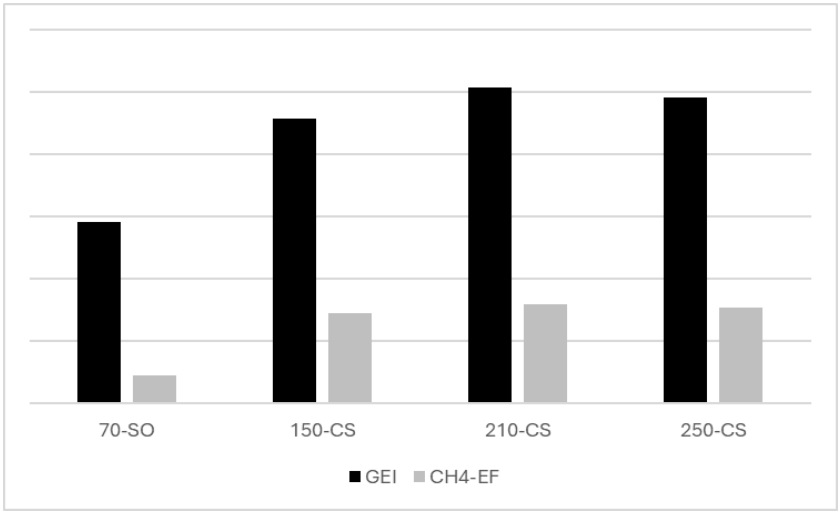

Figure 28. Variations in enteric methane emissions (EF, kg CH_4/head/year) values as a function GEI in the studied groups. *Different superscripts($^{a, b}$) within the bar indicate significant differences at P ≤ 0.05.*

The following table (**Table 11**) shows the physicochemical components of the milk collected in the milk control:

Table 11. Chemical composition (%) milk in the different studied groups

Group	Fat	Protein	BHB	MUFA	PUFA
70-SO	3.94±0.7	3.3±0.3	0.045±0.01	0.96±0.16	0.1±0.02
150-CS	3.25±0.72	3.35±0.32	0.06±0.02	0.74±0.13	0.07±0.02
210-CS	3.8±1.2	3.32±0.3	0.04±0.02	0.85±0.4	0.08±0.03
250-CS	4.6±0.7	3.2±0.2	0.07±0.03	1.05±0.2	0.1±0.03
P-value	*P>0.05*	*P>0.05*	*P>0.05*	*P>0.05*	*P>0.05*

[1]BHB: β-Hydroxybutyrate; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids;

The ANOVA results show that the groups did not differ significantly in terms of the physicochemical components of milk as a function of lipid supplementation. When comparing (*Independent t-test*) enteric methane emissions and milk values we found significant differences

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(P<0.05) and that may be related to the nutritional content of the ration This could be important for dietary planning and recommendations.

The correlations between CH_4-EF and milk parameters are exposed in **Table 12.**

Table 12. Correlations between enteric methane emissions and milk composition (%) milk in the different studied groups

Item	CH_4-EF	Fat		Protein	BHB	MUFA	PUFA
CH_4-EF	1.000	-0.0		-0.107	0.371	-0.253	-0.517
Grasa	-0.0	1.000		-0.966	0.364	0.967	0.856
Proteína	-0.107	-0.966		1.000	-0.591	-0.916	-0.778
BHB	0.371	0.364		-0.591	1.000	0.288	0.141
MUFA	-0.253	0.967		-0.916	0.288	1.000	0.959
PUFA	-0.517	0.856		-0.778	0.141	0.959	1.000

[1]BHB: β-Hydroxybutyrate; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids;

A thorough examination of the correlations reveals three significant findings: Firstly, a positive correlation (0.371) between CH_4-EF and BHB values indicates that an increase in CH_4-EF is associated with a concomitant tendency for an increase in BHB. Secondly, a robust negative correlation coefficient of -0.966 has been observed between the fat and protein contents of milk, suggesting that as the fat content of milk increases, the protein content concomitantly tends to decrease.

Finally, there is a strong positive correlation (0.959) between fat content and MUFA (0.967) and MUFA and PUFA (0.959) suggesting that these two variables tend to increase together.

3.4. DISCUSSION

As demonstrated in this study and as has been previously observed by various authors (Couto *et al.*, 2018), the physicochemical characteristics of each lipid supplement have a substantial impact on the energy content of the ration (GEI). However, these supplements do not influence the other components of the ration. Conventionally, CS has been used in high-production dairy cows to enhance the energy density of the ration without altering rumen dynamics. The saponification of long chain fatty acids, typically derived from palm oils, with calcium ions results in calcium salts, a type of rumen-inert fat containing high levels of fatty acids such as palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2); this was originally proposed by Jenkins and Palmquist (1982). Thus, feeding calcium salts of fatty acids (CS), which are inert in the rumen, can enhance the energy density of rations and has no negative effects on ruminal fermentation.

CSFA is a complex of calcium ions with long-chain fatty acids; the main sources are soybean oil or palm oil, depending on the commercial product. CS is inert in the rumen and is dissociated in the acidic conditions of the abomasum. Among the benefits of CS is the

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possibility of increasing the energy content of the diet without influencing fiber digestibility, allowing high levels of inclusion in ruminant diets. In this sense, CS are interesting, because they do not change ruminal fermentation (Sirohi *et al.*, 2010). Thus, CS is an energy supplement that in combination with other foods can increase dry matter intake, therefore providing good availability of nutrients for satisfactory yields.

The observed variation in CH_4-EF values between the SO group and the other CS farms are significant. The percentage differences between CH_4-EF in the SO farm (44.9 kg CH_4/head/305 DIM)) and the other methane emissions are: 105.72% between SO farm and 150-CS farm, where the average CH_4-EF is 145.6 kg (CH_4/head/305 DIM). 111.79% between SO farm and 210-CS farm where the average CH_4-EF is 158.7 (kg CH_4/head/305 DIM) and 109.48% between SO farm and 250-CS farm where the average CH_4-EF is 153.5 kg CH_4/head/305 DIM). The figures presented demonstrate the magnitude of the disparity between the SO lot and the other groups, expressed in percentage terms. Consequently, a percentage difference of 105.72% between 70-SO and 150-CS signifies that the discrepancy between these two values is approximately 105.72% of the mean value of both. This finding indicates a substantial difference, as the maximum value (145.6kg CH_4/head/305 DIM) exceeds the minimum value (44.9kg CH_4/head/305 DIM). However, when focusing on CS-supplemented farms and enteric methane emissions, a similar trend is observed. The percentages demonstrate an 8.61% discrepancy between the farm that received 150-CS and the farm that received 210-CS and a 5.28% difference between the farm that received 150-CS and the farm that received 250-CS. The relatively low percentage differences (8.61% and 5.28%) indicate that these values are close to each other, suggesting less variability and greater consistency.

Considering the GEI value for each group, we found differences in intake (g/kg/DM as feed) between the four study groups: 14.37% between 70-CS and 150-CS; 5.71% between 70-CS and 210-CS and 4.71% between 70-CS and 210-CS. The relatively low percentage differences (5.71% and 4.71%) among the 70-SO and the 210-CS and 250-CS groups suggest that these DMI values are relatively close, indicating less variability and greater consistency. The estimation of enteric methane emissions is significantly influenced by differences in GEI, particularly between the 70-SO and 150-CS group.

In summary, our results agree with previous studies that have shown to reduce methane production more effectively than calcium soaps derived from palm fatty acids. This is due to the higher content of polyunsaturated fatty acids in soybean oil, which directly impacts methanogenic microorganisms (Woodward *et al.,* 2006)

Soybean oil and calcium salts of palm oil fatty acids (CS) exhibit notable differences in their impact on ruminal fermentation. Soybean oil, a liquid fat, can be directly incorporated into the diet due to its high polyunsaturated fatty acids (PUFAs) content. However, these PUFAs are more susceptible to oxidation and less stable in the rumen. In contrast, calcium salts of palm oil fatty acids are solid, rumen-protected fats more stable in the rumen environment. They are designed to bypass the rumen and be digested in the small intestine.

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Theoretically, these physical characteristics should have a different impact on ruminal fermentation. Specifically, soybean oil supplementation can inhibit fiber digestion, reducing the efficiency of ruminal fermentation due to its direct interaction with rumen microbes. By contrast, calcium salts exhibit minimal impact on ruminal fermentation due to their reduced solubility in the rumen (Couto *et al*., 2018).

The findings of the present study are in alignment with the physicochemical characteristics of the lipid supplements under scrutiny: the use of soybean oil, through its capacity to curtail fiber fermentation, has been shown to impede the activity of methanogens, while calcium soaps, formulated to circumvent the rumen, have been observed to exert minimal influence on the activity of rumen microorganisms, thereby favoring an elevated level of enteric methane (Zahera *et al*., 2024).

Various authors have previously highlighted that whilst the utilization of protected fats enhances nutrient use at the ruminal level (Wulandari *et al.*, 2020; dos Santos Neto *et al.*, 2022), their role as methane inhibitors is questionable (Zahera *et al*., 2024). Conversely, natural oils have the potential to mitigate methane production as they affect fiber digestibility (Patra *et al.*, 2013).

According to Jenkins (1993) dietary fats interfere with rumen fermentation by incorporating lipids into microbial cell membranes, disrupting membrane integrity and cellular function, altering microbial cell attachment to plant surfaces, and affecting the activity of microbial hydrolytic enzymes.

Reduced fibre digestibility is often followed by reduced dry matter intake (DMI) and milk production and therefore it is important to take the CH_4 reduction per kg of DMI or per kg of product into account. Substituting fermentable OM with nonfermentable fat will reduce microbial fermentation and microbial protein synthesis, thereby supplying microbial protein for milk production (Canale et al., 1990).

3.4.1. Fat supplementation and milk composition

Studies have shown that calcium soaps can improve milk yield and composition. For example, calcium soaps from safflower oil have been found to increase milk production without negatively affecting nutrient intake and digestibility (Couto *et al.,* 2024). Our results differ from the above studies in that we found no significant differences in milk quality. This indicates that while supplementation with calcium soaps affects methane emissions, it does not impact the fat, protein, MUFA, and PUFA values of milk.

The negative correlation between fat and protein in milk can be explained by several factors related to milk composition and metabolism. Firstly, milk contains several components, including fat, protein, lactose, and minerals, and the proportion of these components can vary depending on factors such as the animal's diet, genetics, and animal management (García *et al*., 2014). Secondly, the synthesis of fat and protein in milk occurs in the mammary gland and is influenced by different metabolic pathways. In instances where there is an increase in fat

synthesis, there is often competition with protein synthesis due to the availability of precursors and energy (Duque *et al.,* 2013). Finally, an animal's diet can also affect the amount of fat and protein in milk. For example, a diet high in energy may increase fat production, while a diet high in protein may increase protein production in milk (Couto *et al*., 2024). In summary, the negative correlation between fat and protein in milk may be the result of a combination of biological and environmental factors that influence the synthesis and balance of these components.

The correlations between CH_4-EF values and the different physicochemical components of milk, showing the correlation between soybean oil supplementation and β-hydroxybutyrate levels during the lactation phase, reflect the lower energy intake (although compensated by higher consumption). In general, β-hydroxybutyrate (BHB) levels in milk are an important indicator in managing the health and productivity of dairy cows, as they are indicators of subclinical ketosis.

BHB values in milk have been used as indirect markers of ketosis in animals when the diet does not meet the metabolic demands of lactation (Ježek et al., 2017). It is imperative to acknowledge that the inherent nature of BHB measurement in the context of dairy control necessitates its implementation at the herd level, as opposed to the individual level. This is predicated on the understanding that within the framework of control, a proportion of animals are likely to fall outside the range of 0.103 to 0.144 mmol/L ascertained from blood samples (Guadagnini, 2020). The prevalence of ketosis, as measured by dairy control, has been documented in Spain. A study conducted in Galicia (http://www.africorlugo.com/memorias.asp) revealed a 22% prevalence, while a study undertaken in 2016 by Bach and Andreu utilizing blood samples identified a 28% incidence of ketosis across various regions of the peninsula.

In our study, none of the study groups exceeded this range, so the findings suggest that although the energy intake of cows supplemented with soybean oil was lower than that of cows supplemented with calcium salts of palm oil, the higher compensatory intake was able to minimize the effect, without detecting negative effects on milk quality.

The correlations found indicate that the positive correlation might indicate a metabolic link between CH_4-EF and BHB. This could suggest that conditions or factors that increase methane production might also promote the production of ketone bodies. Both methane and BHB are involved in metabolic processes. Methane is produced during anaerobic digestion by certain microorganisms, while BHB is produced during ketosis. The correlation might reflect a shift in metabolic states that favor both methane production and ketone body synthesis.

Bielak *et al.* (2016) suggested that long-chain fatty acids mobilized during early lactation of dairy cows are increasingly used as energy substrates at the expense of acetate. As the synthesis of acetate in the rumen is closely linked to methane production, they hypothesized that decreased acetate utilization would result in lower ruminal acetate levels and thus CH_4 production.

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Understanding the relationship between methane production and BHB levels could have implications for environmental studies, particularly in understanding the metabolic activities of microorganisms in different environments. In this sense, the type of fat supplemented in the diet and its impact on ruminal dynamics is an aspect to consider high BHB levels tend to be associated with higher methane emissions. Our data is in line with Bielak *et al.* (2016) results that show that fat mobilization of the host in lactation is inversely related to ruminal CH_4 production and that this effect is not attributed to different DMI.

Further research could explore the mechanisms underlying the correlation between CH_4-EF and BHB, which might involve studying the enzymes and pathways that regulate their production and utilization. Conducting studies on larger and more diverse populations could help confirm the correlation and understand its implications across different demographic groups.

The lack of correlation between CH_4-EF and milk fat content, indicated by a correlation coefficient close to 0, suggests some potential implications: 1) The factors influencing methane production and fat content are likely independent of each other. This means that changes in one variable do not predict changes in the other. 2) CH_4 and fat might be regulated by different metabolic pathways or physiological processes. For example, methane production could be influenced by microbial activity in the gut, while fat content might be more related to dietary intake and lipid metabolism.

In summary, under practical applications such as nutritional assessments or health monitoring, the lack of correlation suggests that CH_4 and milk fat should be considered independently. For instance, interventions aimed at reducing methane production might not necessarily impact fat content and vice-versa (Benedet *et al*., 2019).

Further studies should consider the analysis of milk fatty acid profiles to evaluate the transfer of dietary fatty acids supplied into milk fat and rumen microbiome analysis warrants further attention to improve our understanding of nutrient digestibility and methane production.

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CHAPTER 4.

Effect of nitrooxypropanol (3-NOP) supplementation in high-producing dairy cows: a pilot study.

4.1. INTRODUCTION

Modifying diets and incorporating feed additives is a cost-effective strategy for reducing enteric CH_4 emissions, making them a potentially superior alternative (Honan *et al*., 2021). The effectiveness of feed additive routines is often linked to variations in feed intake, diet composition, roughage quality, and rumen fermentation requirements (Bannink *et al*., 2023). Feed additives can suppress methanogenic archaea directly or indirectly, leading to reduced CH_4 emissions. A notable example is nitrooxypropanol (3-NOP), a synthetic molecule similar to methyl-coenzyme M, identified as a potential methane inhibitor in the intestines (Duval and Kindermann, 2012; Araújo *et al*., 2023).

The synthesis of 3-NOP involves a chemo-selective reduction process, enhancing the efficacy of calcium channel antagonists (Ogawa *et al.*, 1990). This compound has been tested on various ruminant species, including dairy cows, beef cattle, and sheep (Romero-Pérez *et al.*, 2015). Generally, 3-NOP can reduce enteric CH_4 emissions. A commercial feed additive containing 3-NOP has shown effectiveness in reducing methane production in both small and large ruminant trials (Alemu *et al.*, 2021). Dijkstra *et al.* (2018) demonstrated that 3-NOP can reduce intestinal methane emissions in dairy cattle by over 30% on average. Other studies found that 3-NOP decreases CH_4 emissions in sheep and dairy cattle by up to 60% (Yu *et al*., 2021).

Typically, the rumen molar proportion of acetate decreases with the administration of 3-NOP and other CH_4 inhibitors, while the molar proportion of propionate increases, reducing the acetate-propionate ratio (Haisan *et al.,* 2014). 3-NOP has minimal impact on bacteria and protozoa populations, confirming its specificity in targeting methyl-coenzyme M reductase (MCR) found exclusively in methanogenic archaea (Jayanegara *et al*., 2018).

The effect of 3-NOP on enteric CH_4 emissions has been consistent (Melgar *et al.* 2020; Mshary *et al.*, 2024), but further long-term studies are needed to conclusively determine its effects on milk production and composition in dairy cows, which is crucial for the future adoption of this CH_4 mitigation practice. In fact, more recent available literature has in some cases indicated reductions in DMI and milk production from cows supplemented with 3-NOP (Kjeldsen *et al.*, 2022; van Gastelen *et al.*, 2022).

Based on previous results, this study aims to assess the effects of replacing calcium soaps (based on palm oil) with 3-NOP on enteric methane emissions and evaluate the quality of milk produced.

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4.2. MATERIAL AND METHODS

4.2.1. Selected farms, animals, and husbandry

One Holstein Frisian dairy farm located in Galicia (N.W Spain) was evaluated, with an average of 75 cows. The farm was carefully selected based on the *2019 Refinement to the 2006 IPCC Guidelines for high-producing dairy cattle* (>8500 kg/head/year) that receive diets with over 70% feed quality digestibility (DE%) according to the formula DE (MJ/kg)/GE (MJ/kg) x100 and less than 35% NDF (DMI basis). These high-producing cows have calved at least once and are primarily utilized for milk production.

The selected farm was chosen because of the study carried out in the previous chapter, which was supplemented with calcium soap derived from palm oil. It was found that on this farm, enteric methane production was significantly increased (more than 100%) when compared to another farm supplemented with soya oil.

The farm had an average milk production of 38 kg milk/cow/day, which generates an average annual production (at 305 days) within the standard 11,590 kg milk/head/305 days of lactation. The cohort under consideration consisted of 75 multiparous dairy cows (700 ± 50 kg) with an average dry matter intake (DMI) of 25.5 kg/day at peak lactation (day 150 after calving).

Furthermore, the farm was selected to serve as a representative sample of the standard diets (in the form of TMR rations) fed to dairy cattle on dairy farms in the designated study area (**Table 13**).

Diet was fed *ad libitum* three times a day between 07:00-07:30, 14:00-14:30, and 20:00-20:30 h. TMR samples were collected at the beginning of each stage and sent for chemical analysis to the reference laboratory for all farms (Aira SCG, Lugo https://www.aira.es) using the NDS-Pro software platform (RUM&N Company, Italy RUM&N (https://www.rumen.it), developed in collaboration with the Cornell Department of Animal Science and based on the NRC (2001) standards.

Table 13. Chemical composition (g/Kg DM basis) of cows

	DM[1] (g/kg/DM as feed)	aFNDmo (%)	ADF (%)	ADL (%)	NFC (%)	Starch (%)	CP (%)	EE (%)	Ash (%)
Basal diet	452	322.1	208.8	33.3	298.8	247.5	161.0	33.2	76.8

[1]DM: Dry matter, aNDFmo: Neutral detergent fiber analyzed using a heat stable amylase and expressed inclusive of residual ash, ADF: Acid detergent fiber, ADL: Acid detergent lignin, NFC: non-fiber carbohydrate, CP: Crude protein, EE: Ether extract content

In this study, 75 multiparous cows were divided into two groups. group 1 (the 3-NOP group, n=10) received 3-NOP (Bovaer®, DSM-Firmenich, Germany) with the ration (60 mg/kg feed DM), while group 2 (the CSPO group, n=65) received the same basal diet, supplemented with 150 g of calcium soap based on palm oil.

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One aliquot of each milk sample was placed in tubes with a preservative (azidiol) and these were refrigerated at 4ºC prior analysis of milk fat and total protein in an infrared spectrophotometer(Milko Skan S 50B, Foss Electric, Hillerød, Denmark).

4.2.2. Statistics

Due to the low number of animals tested, non-parametric tests were used (Mann-Whitney U test) for two groups. These tests do not assume a specific distribution of the data and are more suitable for small samples.

The statistical analysis was performed using a *confidence interval analysis*, the principal purpose of which was to estimate the range in which the true population mean is likely to lie. This was achieved by deriving averages and standard deviations. The result of this analysis is an indication of the precision of the estimate.

Also using the mean and standard deviation, hypothesis tests such as Student's t-test were performed to compare the means of the two groups. In this way, it was determined whether the differences observed between the groups were statistically significant ($P<0.05$).

Considering the estimations proposed by different studies (Appuhamy *et al.*, 2016; Jayasundara *et al.*, 2016; Niu *et al.*, 2018) which have contributed to the IPPC Refinement (2019), we assume a Ym (%) value of 5.7 for lactating cows. For cows on each farm in the dry-off phase, the Ym (%) value will be 6.3. Later studies have also followed these assumptions (Niu *et al.*, 2021).

The CH_4-EF for each dairy farm (G-A and G-B) were developed using the Tier-2 approach based on gross energy intake (GEI) and Ym (%) as follows: (Equation 10.21 from the 2019 Refinement to the 2006 IPCC Guidelines):

$$\underline{EF = [GE \times (Ym/100) \times 365 \text{ days})]}$$

$$55.65 \text{ MJ/kg } CH_4$$

where EF is the CH_4 emission factor (kg CH_4/head/305 DIM), and GE is gross energy intake (MJ/ head/day). The factor 55.65 (MJ/kg CH_4) is the energy content of methane.

4.3. RESULTS

The differences found between the study groups concerning enteric methane emissions and milk production are shown in **Table 14.**

Table14. Variations in enteric methane emissions (EF, kg CH_4/head/year) and milk yield (Kg/head/305 DIM) values as a function of supplementation

Group	CH_4-EF	Milk yield
CSPO	145.6±10.5	39±2
3-NOP	109.2±6.7	38±3

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When assessing statistical differences, the *t-statistic* value was 94.53, with a *p-value* < 0.001 indicating a significant difference in methane emissions between the two groups, with 3-NOP showing lower emissions.

Concerning milk yield, the statistical difference showed a *t-statistic* of 8.68 with a *p-value* <0.05. There was also a significant difference in milk production, although it was smaller than methane emissions. These results suggest that 3-NOP is not only more effective in reducing methane emissions but also has a minimal impact on milk production.

The magnitude of the reduction in enteric methane emissions, almost 25% in cows supplemented with 3-NOP, is graphically illustrated in **Figure 29.**

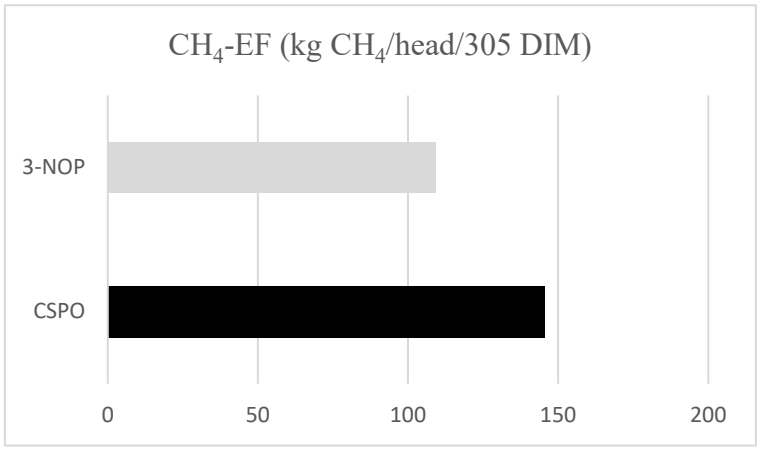

Figure29. Variations in enteric methane emissions (EF, kg CH_4/head/year) values as a function supplementation in the studied group.

The differences found between the study groups regarding enteric methane emissions, supplementation and milk production are shown in **Table 15.**

Table 15. Variations in enteric methane emissions (EF, kg CH_4/head/year) values and mil protein and fat contents in the supplemented groups

Group	CH_4-EF	Fat (%)	Protein (%)
CSPO	145.6±10.5	3.12±0.50	3.28±0.7
3-NOP	109.2±6.7	3.25±0.72	3.35±0.32

A comparison of the 3-NOP and CSPO groups in terms of methane emissions (CH_4-EF), milk fat, and milk protein reveals a statistically significant difference in fat content between the two groups, with cows supplemented with CSPO showing slightly higher fat content.

Furthermore, a t-statistic value of -3.39 and a p-value >0.001 were obtained for milk protein values, indicating that, while there are differences, these are minor compared to the difference in fat. The results suggest that, although both groups have significant differences in terms of fat and protein, the CSPO group tends to have slightly higher values in both components.

To ascertain the correlation coefficient between the fat and protein content in the 3-NOP and RBSA groups, the data were analyzed. This process yielded a coefficient of 0.02, indicating that there is no significant relationship between these two parameters in the groups analyzed. This finding is illustrated in Figure 30.

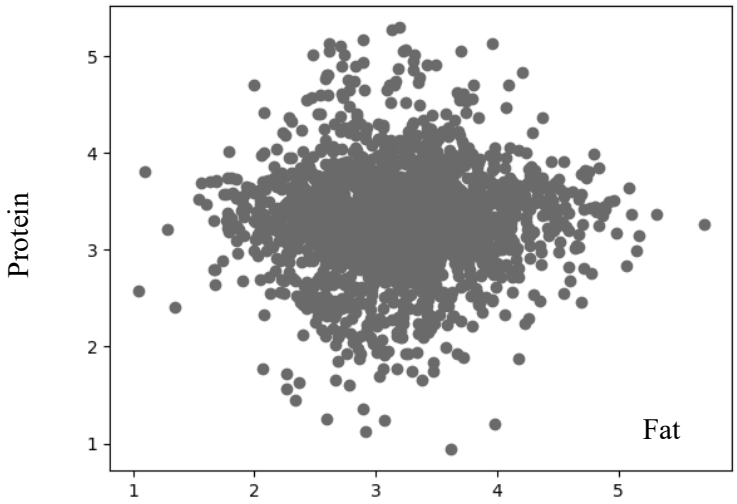

Figure 30. Visual representation of the correlation between fat and protein in the supplemented groups.

By calculating the correlation coefficients between methane emissions (CH_4-EF) and fat and protein contents in the 3-NOP and CSPO groups, it was found that the correlation coefficient between CH_4-EF and Fat is 0.11, while the correlation coefficient between CH4-EF and protein is 0.06. These coefficients indicate a very low correlation between methane emissions and fat and protein contents in the data provided, indicating that there is no significant relationship between these parameters in the groups analyzed, and therefore that variations in methane emissions are not significantly associated with variations in milk fat and protein content.

This can be useful for producers, as they can focus on reducing methane emissions without worrying too much about negatively affecting milk quality in terms of fat and protein.

4.4. Discussion

4.4.1. Effect of 3-NOP supplementation on CH_4-EF emissions

The results of our study are consistent with previous studies highlighting the role of 3-NOP in reducing methane emissions in cattle. Ruminal gram-positive bacteria play a crucial role in the fermentation process, producing acetate, butyrate, lactate, hydrogen, and ammonia,

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which are linked to methanogenesis (Ratti *et al*., 2014; Matthews *et al.*, 2019; Shinkai e*t al*., 2024).

Kinley *et al*. (2020) demonstrated that directly inhibiting CH_4 synthesis can increase H_2 emissions, depending on the extent of CH_4 suppression and the availability of alternative H_2 metabolic pathways. Although ruminal methanogens constitute less than 5% of the rumen microbiota, they are responsible for all methane production in cows (Pitta *et al.,* 2016). The addition of 3-NOP affects the total volatile fatty acid (VFA) concentration, with a slight decrease observed at medium doses of 3-NOP (Alemu *et al.,* 2021a). The rumen microbiota primarily converts bovine feed into VFAs, releasing CO_2 and H_2, which methanogenic archaea then use to produce methane (Knapp *et al*., 2014). Feeding 3-NOP delays methanogenesis in ruminants by increasing H_2 emissions, which is considered an inadequate or unsuccessful formulation (Vyas *et al.,* 2018; Alemu *et al*., 2021b). This is because the availability of H_2 results from reduced methanogenesis due to feeding 3-NOP or other effective methanogenesis inhibitors like monensin and other ionophores. This confirms that methanogens in the rumen convert CO_2 to CH_4 using H_2 generated during rumen microbial fermentation (Morgavi *et al.,* 2010).

Various techniques to reduce CH_4 emissions have been proposed, including adjusting animal management and breeding, refining their diets, and improving feed quality (Hristov et al., 2013). Honan *et al.* (2021) and Arndt *et al.* (2022) examined reducing CH_4, such as adding small feed additives to alter rumen metabolism and decrease methanogenesis. Direct suppression of methanogenesis remains the most effective strategy for reducing intestinal CH_4 emissions (Almeida et al., 2021).

Thus, supplementation with 3-NOP is close to the data observed for supplementation with natural fat sources, such as soybean oil, with emission reductions approaching 25% compared to supplementation with calcium soap oils, the traditional and commonly used supplement in high-producing dairy cattle (Melgar *et al*., 2021).

4.4.2. Effect of 3-NOP supplementation on milk and quality production

The composition of cow milk is influenced by several factors, including breed, stage of lactation, and feeding (Walstra *et al.*, 2005). The rumen microbiota plays an important role in either producing milk components directly or producing precursors used in the mammary gland for milk synthesis, including milk proteins and vitamins. Therefore, alterations in the ruminal environment, along with changes in energy metabolism, and nutrient digestibility by fat, nitrate, and 3-NOP, may affect the microbial synthesis of vitamins and uptake of precursors by the mammary glands, and subsequently the concentrations of proteins and vitamins in milk

Several studies have demonstrated that 3-NOP does not affect milk yield in long-term production studies (Hristov *et al.*, 2015; Van Wesemael *et al*., 2019; Melgar *et al.*, 2020). Although 3-NOP has shown promising results in reducing methane production, its impact on milk production may vary (Melgar *et al.,* 2021; Mshary *et al.*, 2024). In this study, while milk

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production declined in the CSPO-supplemented cohort, this decline was not as marked as the decline observed in methane emissions.

This finding is consistent with other studies indicating that 3-NOP supplementation can lead to a slight decrease in milk production (Baumont, 2018; Hanbeen *et al.*, 2020). These differences may be attributable to various factors, such as the type of diet or farm management. It is imperative to meticulously control and manage their use to optimize benefits while minimizing any potential negative impact on milk production.

Another aspect is milk composition, such as fat or protein. There have been inconsistent reports of the effects of 3-NOP on milk components in dairy cattle. Approximately one-third of dietary protein is utilized by bacteria and protozoa and converted into microbial protein, which provides the majority of AA needed for milk protein synthesis in the mammary glands (Stelwagen, 2003).

The decrease in milk fat observed in dairy cows following 3-NOP supplementation can be attributed to changes in ruminal fermentation patterns, specifically alterations in the following aspects: 1) Alteration of ruminal fermentation: 3-NOP has been shown to inhibit the enzyme methyl-coenzyme M reductase, which is crucial for methane production in the rumen. This inhibition causes a shift in rumen fermentation, increasing propionate production at the expense of acetate and butyrate (Melgar *et al.,* 2021). Since acetate and butyrate are primary precursors of milk fat synthesis, their reduction can lead to lower milk fat content (van Gastelaan *et al.,* 2024); 2) Hydrogen accumulation: Inhibition of methane production leads to increased levels of hydrogen in the rumen. Excess hydrogen has been shown to interfere with normal fermentation processes, affecting the production of volatile fatty acids (VFA), such as acetate and butyrate, essential for milk fat synthesis (Melgar *et al.,* 2021). Thirdly, there is the issue of feed efficiency: Although 3-NOP improves overall feed efficiency and reduces methane emissions, the change in VFA production can lead to a slight decrease in milk fat percentage (Van Gastelaan *et al.,* 2024).

Concerning protein content, although Reynolds *et al*. (2014) observed an increase in milk content, the decrease in this parameter associated with 3-NOP supplementation can be linked to several factors related to changes in rumen fermentation and nutrient utilization: 1) altered rumen fermentation: 3-NOP has been shown to inhibit methane production by targeting methanogens in the rumen, which can lead to a shift in the production of volatile fatty acids (VFAs). These are crucial for microbial protein synthesis.2) Reduction in certain VFAs, such as acetate and butyrate, can impact the overall protein synthesis in the rumen.3) nutrient partitioning: the energy saved from reduced methane production might be redirected toward other metabolic processes rather than milk protein synthesis, which can result in a slight decrease in milk protein content. This, in turn, can affect the synthesis of amino acids and other precursors necessary for milk protein production. These factors highlight the complex interactions between rumen fermentation, nutrient utilization, and milk composition when using 3-NOP. Approximately one-third of dietary protein is utilized by bacteria and protozoa and

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converted into microbial protein, which provides the majority of AA needed for milk protein synthesis in the mammary glands (Stelwagen, 2003)

Despite the limitations of the study, which included the administration of 3-NOP supplementation on a single commercial farm, on a specific diet, and with a limited number of animals, the results obtained allow us to suggest that 3-NOP is a viable candidate as a feed additive because of its strong CH_4 mitigation effects, regardless of animal type and experiment period, without adverse effects on animal performances. The magnitude of the NOP supplementation effect was varied with animal types. Thus, further research will be needed to identify the relationship between NOP supplementation and dietary content (starch, non-fiber carbohydrates, and NDF). These changes highlight the importance of carefully managing 3-NOP supplementation to balance methane reduction benefits with potential impacts on milk composition.

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CONCLUSIONS

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Following the execution of the experimental studies and the consideration of the objectives proposed in this doctoral thesis report, the following conclusion can be drawn:

1. Following the Tier -2 model, recommended by the *2019 Refinement to the 2006 IPCC Guidelines,* the emission of enteric methane (CH_4-EF, kg CH4/head/year) from dairy cows is significantly influenced by dry matter intake, physiological stage, and diet composition, particularly the content of fats. High-producing cows emit greater quantities of methane, especially during peak lactation. However, implementing dietary strategies involving higher fat content can potentially mitigate these emissions. The methane emission factors (CH4-EF, kg CH4/head/year) defined following the TIER-2 model are based on the lactation phase and extend to encompass the entire production cycle (lactation and drying phase). The findings of the study indicate that the data provided by the IPCC are overestimated and should focus exclusively on the lactation phase. According to this criterion, the animals under study exhibit lower values of enteric methane emissions than those established for developed countries. This finding calls into question the role of the bovine sector in the context of climate change, and the necessity of seeking measures to minimize methane emissions, given that its synthesis in ruminants is an energetic cost to the animal's metabolism, to the detriment of other productive functions.

2. The methane emission factors (CH_4-EF, kg CH4/head/year) defined following the TIER-2 model are based on the lactation phase and extend to encompass the entire production cycle (lactation and drying phase). These findings indicate that the data provided by the IPCC are overestimated and should focus exclusively on the lactation phase. According to this criterion, the animals under study exhibit lower values of enteric methane emissions than those established for developed countries. This finding calls into question the role of the bovine sector in the climate change, and the necessity of seeking measures to minimize methane emissions, given that its synthesis in ruminants is an energetic cost to the animal's metabolism, to the detriment of other productive functions.

3. The energy content of the ration (fat or ether extract content) affects enteric methane emissions. the type of fat supplement determines the variations in enteric methane emissions (CH_4-EF, kg CH4/head/year). The potential for applying lipid feed such as soybean oil as a methane mitigation strategy is high because these sources represent "natural" lipid additives, more appreciated environmentally and by consumers. Soybean oil in the diet significantly reduced methane emissions compared to calcium soap salts regardless of the dose.

4. Considering the lipid supplementation traditionally administered to the farms in the study area, soybean oil supplementation is preferable to calcium soap supplements, without negatively affecting the physicochemical quality of the produced milk (protein, fat, polyunsaturated or monounsaturated fatty acids). A positive correlation with ketone body levels (beta-hydroxybutyrate) has been observed, likely due to the lower energy

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contribution of soybean oil. This correlation could be an early biomarker indicating that the administered diet has methanogenic potential.

5. In the search for new supplemental fat sources to minimize enteric methane emissions, the use of nitrooxypropanol (3-NOP) as a substitute for soybean oil reduces around 25% enteric methane emissions compared to supplementation with calcium soap oils, the traditional and commonly used supplement in high-producing dairy cattle. Nevertheless, this supplement, under the conditions of our study caused a slight milk yield, fat, and protein decrease, attributable to diet composition and management of the farm, although not at the same magnitude as enteric methane emissions.

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The study aims to develop a Tier-2 model for greenhouse gas (GHG) inventories under on-farm conditions, using data from diverse dairy production systems in Galicia. This model will align with international standards while addressing the region's production systems variability.

Five objectives are established:

1. Quantify methane emissions from high-production dairy farms, applying the Ym value (%) recommended by the 2019 Refinement to the 2006 IPCC Guidelines.
2. Assess how emission factors (EF) vary depending on physiological stage and milk production
3. Investigate the influence of dietary composition on methane emission factors.
4. Identify physicochemical components in feed rations that could reduce methane emissions under practical farming conditions.
5. Evaluate the impact of lipid supplementation on methane emissions