

A holistic approach to greenhouse gases and ammonia emissions for the sustainability of dairy cattle production

Ana Rodrigues Alves

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Ana Raquel Ferreira Rodrigues Alves



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**A HOLISTIC APPROACH TO GREENHOUSE GASES AND
AMMONIA EMISSIONS FOR THE SUSTAINABILITY OF DAIRY
CATTLE PRODUCTION**

Tese de Candidatura ao grau de Doutor em
Ciência Animal, Especialidade Nutrição,
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de Abel Salazar da Universidade do Porto.

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AO MEU PEQUENO MANUEL

Declaração de Honra

Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.

Porto, 24 de julho 2025

Ana Raquel Ferreira Rodrigues Alves

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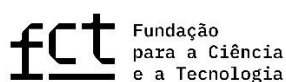
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Resumo

O sector agroalimentar enfrenta o duplo desafio da necessidade de aumentar a produtividade, tendo por base a previsão de um aumento substancial da procura de alimentos, assim como o das alterações climáticas e a obrigatoriedade da redução das emissões gasosas que estas implicam. Os sistemas de produção de ruminantes enfrentam desafios específicos em termos de gases com efeito de estufa (GEE) para além do dióxido de carbono (CO_2), como o metano (CH_4) e o óxido nitroso (N_2O), associados à fermentação entérica e à gestão dos efluentes pecuários. Paralelamente, os ruminantes convertem biomassa vegetal, inviável para alimentação humana, em produtos de alto valor nutricional, e em fertilizantes orgânicos. Por conseguinte, fatores de emissão precisos que tenham em conta o clima mediterrânico, bem como soluções de mitigação que contemplem o equilíbrio necessário entre a redução das emissões gasosas e a produção de proteína, são essenciais.

A presente tese de doutoramento pretende contribuir para uma compreensão mais detalhada das emissões gasosas das instalações de vacas leiteiras portuguesas, bem como avaliar estratégias de mitigação, nomeadamente através de uma nova abordagem nutricional com a inclusão de biochar. Especificamente, esta tese tem como objetivos: i) explorar os efeitos do biochar como aditivo alimentar para reduzir a produção de CH_4 proveniente da fermentação entérica; ii) avaliar o potencial das emissões gasosas dos dejetos de vacas leiteiras, num piso simulado, a partir de diferentes sistemas de alimentação, fases de lactação e tempos de amostragem; iii) medir as emissões de CH_4 e amoníaco (NH_3), identificando as variações sazonais e diárias em instalações naturalmente ventiladas de vacas leiteiras.

Para o estudo da inclusão do biochar optou-se por uma abordagem combinada: realizou-se uma revisão dos estudos *in vitro* e *in vivo* existentes na literatura sobre o potencial de redução do CH_4 entérico (Capítulo Dois); e um estudo experimental utilizando dois tipos de biochars, a dois níveis de inclusão, testados em incubações *in vitro* de 24 horas (Capítulo Três).

Os resultados indicaram a necessidade de metodologias mais padronizadas para facilitar a comparabilidade, bem como a avaliação de características específicas do biochar, como polaridade, a aromaticidade, teor em cinza, entre outras. Mais estudos são também essenciais para compreender o mecanismo pelo qual o biochar afeta a produção de CH_4 , bem como para avaliar o impacto da inclusão do biochar nas comunidades microbianas (Capítulo Dois).

Alguns destes aspetos foram posteriormente confirmados pelo ensaio experimental (Capítulo Três): a inclusão de biochar, independentemente do tipo de biomassa

(agroflorestal e casca de batata) e do nível de inclusão (5 ou 10% de matéria seca), aumentou a produção de CH_4 de 10.1 para 17.2 %, comparativamente ao controlo (sem a inclusão de biochar), e alterou o padrão de fermentação ruminal. Isto indica que a temperatura de pirólise e a área superficial são parâmetros insuficientes para caracterizar e prever o desempenho do biochar, pelo menos em testes *in vitro*.

Relativamente à melhoria dos fatores de emissão para as instalações de vacas leiteiras em Portugal, seguiu-se também uma dupla abordagem: numa primeira fase, foi dado foco ao potencial de emissão da decomposição dos dejetos num sistema de câmaras em laboratório (LCS) que simulam o piso das instalações, durante 48 h (Capítulo Quatro). Este estudo avaliou o efeito dos sistemas de alimentação, do estado de lactação e dos tempos de amostragem no potencial de emissões gasosas dos dejetos de vacas leiteiras (Capítulo Quatro); numa segunda fase, realizou-se uma monitorização a longo prazo (2 anos, 4 estações cada) das emissões de CH_4 (estudadas pela primeira vez em Portugal) e NH_3 de três diferentes instalações de vacas leiteiras naturalmente ventiladas (Capítulo Cinco). Este trabalho contribui não só para uma avaliação geral dos fatores de emissão de CH_4 e NH_3 , como também demonstrou os padrões sazonais e diários de emissões das instalações de vacas leiteiras.

No estudo com LCS (Capítulo Quatro), os tratamentos incluíram a avaliação do efeito do sistema de alimentação [alimento completo (TMR), alimento completo mais concentrado no robot (TMR + robot) e alimento completo mais concentrado em alimentadores automáticos (TMR + AF)], estágio de lactação (vacas no pico e pós-pico) e tempos de amostragem [ordenha da manhã (T8h), no meio do dia (T12h) e antes da ordenha do fim do dia (T17h)] no potencial de emissões gasosas dos dejetos das vacas leiteiras.

Os resultados do estudo de LCS demonstraram que as emissões cumulativas de $\text{N-N}_2\text{O}$ foram mais elevadas no sistema TMR + robot, bem como nas vacas após o pico de lactação e no tempo de amostragem T17h. Detetou-se uma interação entre o estágio de lactação e o tempo de amostragem dos dejetos para as emissões de N-NH_3 e $\text{N-N}_2\text{O}$ (g/kg de N orgânico solúvel). Além disso, os dejetos das vacas após o pico de lactação originaram também as maiores emissões cumulativas de N-NH_3 (Capítulo Quatro).

No estudo de longa duração desenvolvido no Capítulo Cinco, recolheram-se as amostras de ar em intervalos de dois minutos em cinco pontos interiores das instalações de vacas leiteiras através de um multiponto (modelo INNOVA 1409-12, Lumasense Technologies, Ballerup, Dinamarca) conectado a um monitor de gás fotoacústico (modelo INNOVA 1412i-5, Lumasense Technologies).

A medição revelou um aumento das emissões de CH₄ e NH₃ ao longo do dia, com elevada variabilidade intra-horária. Além disso, obtiveram-se os fatores de emissão para o CH₄ e para o NH₃ de 309,5 e 30,6 g/dia/cabeça normal, respetivamente (Capítulo Cinco).

As vias mais promissoras para futuros estudos de investigação abordadas em detalhe nesta tese incluem explorar: 1) o potencial efeito cascata do biochar, 2) aditivos alimentares mais promissores para reduzir as emissões gasosas provenientes do piso das instalações, e 3) estudos adicionais de longa duração para monitorizar as emissões de GEE e NH₃ em instalações naturalmente ventiladas de vacas leiteiras.

Em suma, esta tese de doutoramento contribui para uma abordagem holística que se alia aos esforços contínuos do setor leiteiro nos últimos anos. Além disso, acrescenta conhecimento relativo à redução e monitorização das emissões gasosas de instalações de vacas leiteiras, nomeadamente através de estratégias alimentares, e da avaliação das emissões de GEE e NH₃ dos dejetos de vacas leiteiras e das suas instalações.

Em conclusão, os trabalhos da presente tese contribuem para melhorar os fatores de emissão, tornando-os mais atualizados e específicos, visando assegurar um futuro próspero e competitivo para o setor leiteiro.

Keywords: Instalações para vacas leiteiras, Metano, Amónio, Biochar, *In vitro*, Fatores de emissão

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Abstract

The agrifood sector faces the dual challenge of increased productivity needs, as food demand is expected to increase substantially as well as the great challenge that climate change and required emission reduction entail. Ruminants sector has specific challenges in terms of in terms of greenhouse gases (GHG) other than carbon dioxide (CO₂), such as methane (CH₄) and nitrous oxide (N₂O) linked to enteric fermentation and manure management. Simultaneously, these animals convert plant biomass into human-edible, nutrient-rich products and organic fertilizer. Therefore, precise emission factors that account for Mediterranean climate as well as mitigating solutions taking into consideration the necessary balance between emission reduction and protein production are paramount.

This PhD thesis aims to contribute towards a more precise understanding on gaseous emissions from Portuguese dairy cows' barns, as well as evaluating mitigating strategies, namely a novel nutritional scheme based on biochar. More specifically, this thesis has the objectives of: i) to explore the effects of biochar as feed additive to reduce CH₄ production from enteric fermentation, ii) to assess gaseous emissions potential of dairy cow excreta, in a simulated barn floor, from varying feeding systems, lactation and sampling times and iii) to measure on-farm CH₄ and ammonia (NH₃) emissions identifying seasonal and daily variations in naturally ventilated dairy cattle barns.

A combined approach was used for the dietary biochar inclusion. A review of existing *in vitro* and *in vivo* studies in the literature was made regarding the potential for enteric CH₄ reduction (Chapter Two); and an experimental study using two types of biochar, at two inclusion levels, tested in *in vitro* 24-h incubations (Chapter Three).

Results indicate the need for more standardized methodologies to improve comparability, as well as proper evaluation of the biochar characteristics, such as polarity, aromaticity, and ash content, among others. Further research is also essential to understand the mechanism by which biochar reduces CH₄ production, as well as to assess the impact of biochar inclusion on microbial communities (Chapter Two).

Some of these aspects were further confirmed by the experimental studies (Chapter Three): biochar inclusion, regardless of the source (agroforestry and potato peel residues) and level (5 or 10% of dry matter), increased CH₄ production by 10.1 to 17.2 %, in comparison to no additive application, and changed the fermentation pattern. This indicates that pyrolysis temperature and surface area are not sufficient as characterization parameters and predictions of biochar's performance at least when testing *in vitro*.

Regarding improvements to the emission factors of Portuguese dairy cattle barns, a two-pronged approach was also designed: In one hand, focus was given to 48-h emission potential from excreta decomposition in laboratory chamber system (LCS) simulating the

barn floor (Chapter Four). On the other hand, a long-term monitoring (2 years, 4 seasons each) of CH₄ (studied for the first time in Portugal) and NH₃ emissions from three different naturally ventilated dairy cattle barns was performed (Chapter Five). This work not only contribute to an overall assessment of CH₄ and NH₃ emission factors, but also demonstrate the seasonal and daily emissions potential from dairy cows' barns.

In the LCS study (Chapter Four), treatments included the evaluation effect of the feeding system [total mixed ration (TMR), total mixed ration plus concentrate at robot (TMR + robot), and total mixed ration plus concentrate in automatic feeders (TMR + AF)], stage of lactation (peak and post peak cows) and sampling times (a.m. milking (T8h), at middle day (T12h) and before p.m. milking (T17h)] on gaseous emissions potential from dairy cows' excreta.

Results demonstrate that cumulative N–N₂O emissions were the highest in TMR + robot, as well as in post peak cows and sampling time T17h. An interaction between stage of lactation and sampling time was detected for N–NH₃ and N–N₂O (g/kg organic soluble N) emissions. Additionally, post peak cows also produced the highest cumulative N–NH₃ emissions (Chapter Four).

In the second prong (Chapter Five) air samples were collected at intervals of 2 min in five indoor points at each dairy barn a multipoint (model INNOVA 1409–12, Lumasense Technologies, Ballerup, Denmark) connected to a photoacoustic gas-monitor (model INNOVA 1412i-5, Lumasense Technologies).

Results indicate that CH₄ and NH₃ emissions increased in the daytime, with high intra-hour variability. Besides, the emission factors obtained of CH₄ and NH₃ were 309.5 and 30.6 g/day/livestock unit, respectively (Chapter Five).

The most promising avenues for future research addressed with detail in this PhD thesis include the exploitation of: 1) the potential cascade effect of biochar, 2) the most promising feed additives to reduce gaseous from the barn floor, and 3) additional long-term studies to monitor GHG and NH₃ emissions from naturally ventilated cattle barns.

Overall, this PhD thesis contributes to a holistic approach to the ongoing efforts of the milk sector in recent years. Furthermore, add knowledge on the reduction and monitoring of gaseous emissions from dairy cows' barns namely through nutritional strategies to reduce enteric CH₄ emissions, and evaluation of GHG and NH₃ emissions from cows' excreta and the whole barn. In conclusion, these works provide more up-to-date emission factors, ensuring a prosperous and competitive future for the milk sector.

Keywords: Dairy cattle barns, methane, ammonia, biochar, *in vitro*, emission factors

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List of Abbreviations

3-NOP- 3-Nitrooxypropanol

ADFD - Acid detergent fiber digestibility

AFB - Agroforestry biochar

APA - Portuguese Environment Agency

C- Carbon

CH₄ – Methane

CH₄I_M – CH₄ emissions per unit of milk

CH₄I_g – CH₄ emissions per unit of gain

CLRTAP - Convention on long-range transboundary air pollution

CO₂ - Carbon dioxide

CO₂-eq - Carbon dioxide equivalent

CoM - Methyl-coenzyme M

CoM-S-S-CoB - Heterodisulfide of coenzyme M and coenzyme B

COP21 - 21st Conference of the Parties

CP - Crude protein

CPD - Crude protein digestibility

CS - Corn silage

DM - Dry matter

DMD - Dry matter digestibility

DMI - Dry matter intake

EFs - Emission factors

EU - European Union

FD - Fiber digestibility

FI - Feed intake

GHG - Greenhouse gases

GWP - Global warming potential

H₂ - Hydrogen

HS – Haylage

IPCC - Intergovernmental Panel on Climate Change

LAB - Liquid associated bacteria

LCS - Laboratory chambers system

LU - Livestock unit

MCR - Methyl-coenzyme M reductase

MY – Milk yield

N - Nitrogen

N₂O - Nitrous oxide
NDF - Neutral detergent fiber
NDFD - Neutral detergent fiber digestibility
NH₃ - Ammonia
NH₃-N - Ammonia-N
NH₄⁺ - Ammonium ion
NIR - National Inventory Report
NO₃⁻ - Nitrate
OM - Organic matter
P - Phosphorus
pH_{PZC} - pH at the point of zero charge
PM2.5 - Fine particulate matter
PPB - Potato peel biochar
RH – Relative humidity
S - Sulphur
S_{BET} - Surface area
SDG - Sustainable Development Goals
SEM - Standard error of the mean
T8h - Sampling time before a.m. milking
T12h - Sampling time at middle day
T17h - Sampling time before p.m. milking
TMR - Total mixed ration
TMR + AF - Total mixed ration plus concentrate in automatic feeders
TMR + robot - Total mixed ration plus concentrate at robot
UN - United Nations
UNN - Urinary urea-N
UNECE - United Nations Economic Commission for Europe
UNFCCC - United Nations Framework Convention on Climate Change
VFA - Volatile fatty acids
V_{meso} - Mesopore volume
V_{micro} - Micropore volume
V_{Total} - Total pore volume
WG - Weight gain

Chapter One

General Introduction

Context

Rethinking food systems to improve agrifood sector sustainability is crucial in a scenario where the worldwide population will reach 9.7 billion by the year 2050, and food demand will increase by 50% compared to 2012 [1]. Growing even faster than worldwide population, the global minimum dietary requirements increased by around 29%, and food consumption by over 37% (based on billion kcal/day) between 2000 and 2020 [2]. Yet approximately 10% of the worldwide population was undernourished by 2021, and 11.7% had no guarantee of food safety by 2020 [2].

Inevitably, population pressure and consumption growth doubled the annual expansion rate of global cropland from 2003 to 2019, and increased cropland area by 9% [3], encouraging the adoption of innovative strategies to preserve biodiversity, soil health and fertility, water quality, and promoting sustainable development [4]. Rebuilding soil organic matter (OM) is crucial to ensure food security and enhance carbon (C) sequestration, with the agricultural sector playing a central role as a driver for transition in agrifood sector [5].

The Rome Declaration on Nutrition and the Framework for Action on Nutrition was adopted in 2014 by 193 countries [6], to work on solutions to tackle malnutrition and eradicate hunger. Moreover, the Sustainable Development Goals (SDGs) were adopted in 2015 by United Nations (UN) including 17 SDGs and 169 targets connecting the three dimensions of sustainability: environmental, social, and economic [7]. To accelerate the implementation of policies and programs to eliminate malnutrition in all its forms the UN Decade of Action on Nutrition was created as a commitment by United Nations Member States, following the recommendations of the International Conference on Nutrition Framework for Action and the UN SDGs by 2050 [8].

Food insecurity has gained a new perspective in European (EU) countries with uncertainty in global trade caused by the COVID-19 pandemic and the Russian military aggression against Ukraine. This transition was particularly challenging but at the same time it enabled the exploration of alternative approaches for global agricultural markets [9]. Simultaneously, the global sense of urgency about climate change pressures decision-makers to define mitigation policies for all economic sectors. Concerns about the effect of atmospheric greenhouse gases (GHG) concentrations increase on climate change date back to the 19th century, with the scientific community reaching a consensus in recent decades on the contribution of anthropogenic actions to global warming from the mid-20th century [10, 11]. The Kyoto Protocol was the first international treaty signed by parties to the United Nations Framework Convention on Climate Change (UNFCCC), first adopted in 1997 by 192 parties, having the global objective of reducing GHG

emissions by at least 5% below 1990 levels [12]. A second commitment for the 2012-2020 period was signed by 147 parties with a 20% reduction target below 1990 levels [13].

For the continuous effort to “significantly reduce the risks and impacts of climate change,” the UNFCCC signed The Paris Agreement at the 21st Conference of the Parties (COP21), held at Paris in 2015, in which nations involved compromise to make common efforts to keep global temperature century below 2 degrees Celsius above pre-industrial levels, pursuing to limit even further to 1.5 degrees Celsius. Countries were divided into three main groups: Annex I, Annex II, or Non-Annex-I, according to their level of commitment. Parties to Annex I (including Portugal) must submit annual inventories with accomplishments about air pollutants from all sectors. This national reports follows the Intergovernmental Panel on Climate Change (IPCC) methodologies and includes all air pollutants considered by UNFCCC, the convention on long-range transboundary air pollution (CLRTAP), EU regulations - (EU) 525/2013 of the European Parliament and of the Council of 21 May 2013, (EU) 749/2014 of 30 June 2014, and the Directive (EU) 2016/2284 of the European Parliament and the Council of 14 December 2016, on the reduction of national emissions of certain atmospheric pollutants, amending Directive 2003/35/EC and repealing Directive 2001/81/EC.

In Portugal, the National Inventory Report (NIR) on the GHG emissions is responsibility of the Portuguese Environment Agency (APA), a public institute. For this purpose, the Resolution of the Council of Ministers number 68/2005 created the National System for the Estimation of Emissions by Sources and Removals by Sinks of Air Pollutants to accurately evaluate the emissions by sources and removals by sinks of air pollutants. Considering livestock production, the NIR on GHG emissions assesses methane (CH₄) emissions from enteric fermentation and manure management, and direct and indirect nitrous oxide (N₂O) emissions from soils and manure management. The emissions are calculated for different animal type and category according to IPCC guidelines, with methodological complexity varying based on each country's availability [14].

In 2022, in Portugal, the agriculture sector accounted for 12.3% of the national GHG emissions, which represents a 5.3% decrease compared to 1990 [15]. Of the total emissions from agriculture, CH₄ contributed 72.0% while N₂O contributed 27.6% (expressed in CO₂-eq) [15]. Enteric fermentation and manure management were responsible for 80.2% and 16.6%, respectively, of the total CH₄ from the sector. Direct and indirect emissions from agricultural soils and manure management were the primary contributors for N₂O emissions, with 89.4% and 10.5%, respectively [15]. Non-dairy cattle were the largest contributors to CH₄ emissions from enteric fermentation, responsible for

57.2%, followed by dairy cattle at 21.7%, and sheep at 15.9%. In terms of manure emissions, swine manure was the primary source contributing 55.6%, followed by dairy cattle at 29.5% and poultry at 11.1% [15]. Most direct N₂O emissions from the agriculture sector originated from solid manure storage, which represented 82.9% of the total. Within this category, manure management from poultry was responsible for 52.6%, while cattle for 37.0% [15]. According to the same report, a significant majority (97.7%) of indirect N₂O emissions resulted from the loss of volatile nitrogen (N), in the form of ammonia (NH₃) and nitrogen oxides, during manure collection and storage. Additionally, N lost through runoff and leaching from solid storage of manure into soil contributed to these emissions [15].

The United Nations Economic Commission for Europe (UNECE) and CLRTAP along with its related Protocols requires Parties to annually update their inventories of relevant pollutants, such as NH₃. The National Emissions Ceiling Directive (EU) 2016/2284 on the reduction of national emissions of certain atmospheric pollutants, sets forth new obligations aimed at aligning the European Union (EU) and its Member States with international commitments under the CLRTAP. This includes specific adherence of Protocol to Abate Acidification, Eutrophication and Ground-level Ozone, which was originally established in 1999 and revised in 2012, commonly referred as the “revised Gothenburg Protocol”. In 2022, the agriculture sector was responsible for 91.1% of the national NH₃ emissions [15]. Organic fertilizers are responsible for 25.3%, urine and dung deposited by grazing animals for 21.0%, and poultry for 19.2% and cattle for 14.4% of those total emissions [15].

Ruminant livestock play a crucial role in achieving the goal of zero hunger by converting plant biomass into high-quality, human-edible protein [7]. They contribute significantly to global food security, ecosystems, and climate. However, they are also responsible for GHG emissions, with enteric CH₄ at the forefront of concerns [16]. Therefore, it is essential to ensure the sustainability of the livestock sector by establishing accurate GHG and NH₃ emissions mitigation plans, including strategies with recognized effectiveness of reduction.

Gaseous emissions from dairy cattle farms

The impact of livestock sector on human nutrition, the economy, ecosystems, and the environment is significant and undeniable [17]. Milk and dairy products are rich in carbohydrates, lipids, proteins, water, vitamins, and minerals [18]. In 2023-year, the EU produced 160.8 million tonnes of raw milk, of which 98% was cow's milk [19], with Portugal contributing 1 996 212 tons [20]. Globally, dairy cattle production has grown substantially in recent decades, with projections indicating an additional increase between 2024 and 2033 [21].

Manure from livestock production applied to croplands, is a valuable source of OM, N, phosphorus (P), and other essential elements required for plant growth [22]. Therefore, the properly use of manure as organic fertilizer not only reduces inorganic fertilizer use and cost, but also improves soil health, and OM, and enhances soil C sequestration [23]. The ruminant sector is also an important source of direct and indirect gaseous emissions to the atmosphere, and significant losses of N and P fractions from manure to the subsoil, the surface, and groundwater (Figure 1). In the year 2022 CH₄ emissions from enteric fermentation and N₂O emissions from soils were responsible for 79% of total emissions from EU's Agriculture [24]. Additionally, in the same year the agriculture sector was responsible for 93% of total NH₃ emissions from EU [25].

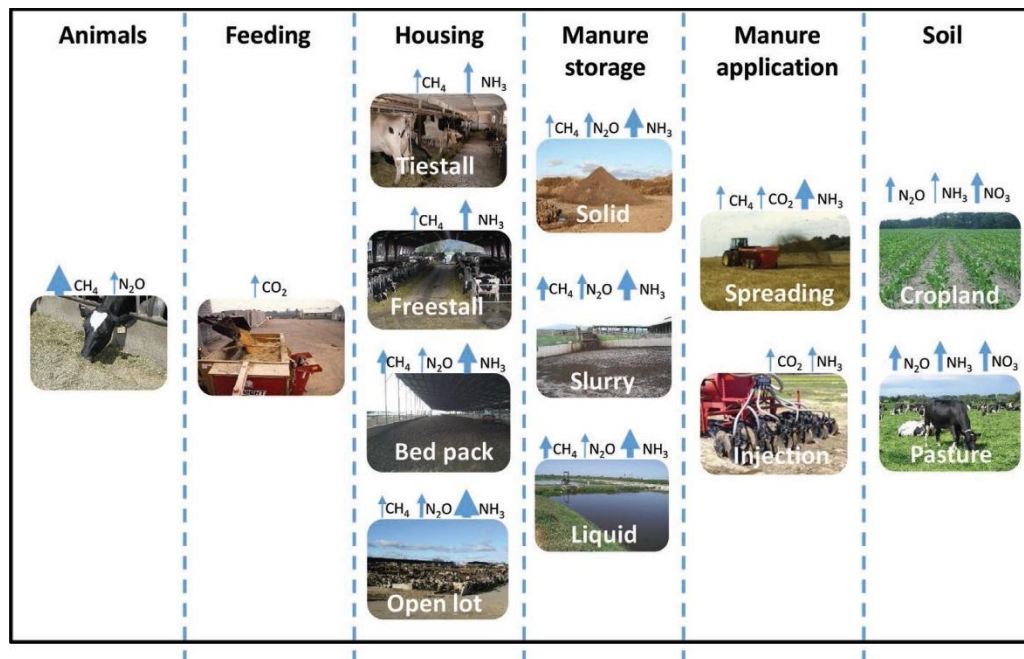


Figure 1 - Direct and indirect gaseous sources emitted from dairy cattle farms. Relative amounts are represented by different size arrows (Reprinted with permission from [26]) (CC BY-NC-ND 3.0).

Different emission sources can be found at dairy cattle farms. The animal release CH_4 and N_2O emissions, manure and bedding decomposition along with manure storage produce NH_3 , CO_2 and N_2O emissions. Manure applied to cropland contribute to CH_4 , CO_2 and NH_3 , while direct emissions from soils, and manure from pasture animals release N_2O , NH_3 and nitrate (NO_3^-) (Figure 1) [26].

Methane is the second most important anthropogenic GHG after CO_2 with a half-life of 12.4 years. On a 100-year time horizon, CH_4 has a global warming potential (GWP) of 28 larger than CO_2 [27-29]. According to Jackson *et al.* [28], CH_4 levels are now 2.6-fold higher than in pre-industrial times. Methane produced from enteric fermentation varies with factors such as forage type and quality, proportion of dietary concentrates, animal breed, among others [29, 30]. The mechanism of enteric CH_4 production will be discussed in the upcoming section titled “Enteric methane emissions”.

Nitrous oxide has a GWP 265 times that of CO_2 (on a 100-year time horizon), contributing to respiratory diseases, acid rain, and the eutrophication of soil and surface waters [2, 31, 32]. Nitrous oxide released as a byproduct of the microbial fermentation has a minimal contribution to total barn N_2O emissions [33]. Ammonia and N_2O emissions from agricultural soils and from manure are influenced by the availability of ammonium (NH_4^+), NO_3^- and labile C compounds. These emissions are also regulated by soil redox conditions and physicochemical properties [34, 35]. In the atmosphere, NH_3 can be rapidly converted to NH_4^+ (an aerosol constituent), reacts with sulfuric and nitric acid forming fine particulate matter ($\text{PM}_{2.5}$). Additionally, can lead to acidification and eutrophication and is an indirect source of N_2O emissions [31, 36].

N emissions from dairy cattle barns

Ammonia is produced in the rumen from the degradation of dietary proteins and other N compounds, and can be used by rumen microbes to synthesize microbial protein [38] (Figure 2). The protein type, ruminal dilution rate, ruminal pH, and predominant microbial population affect protein degradation [38]. If a deficit of protein occurs NH_3 is recycled through rumen wall or recycled via the saliva. On the other hand, if the protein amount surpasses rumen microorganisms' requirements, it is degraded and the excess of ruminal NH_3 is released into the blood, metabolized by the liver, and synthesize into urea. Then, can be recycled via the rumen wall and across epithelium through saliva, or filtered by the kidneys and excreted in the urine, and to a lesser extent, in milk and faeces [38, 39].

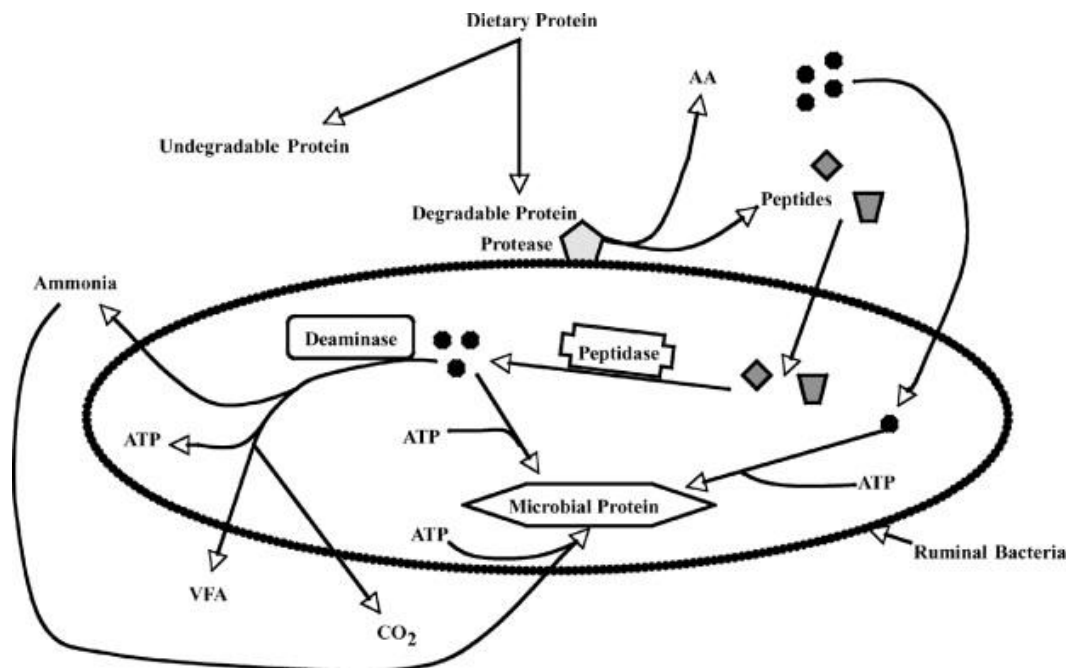


Figure 2 - Schematic representation of protein degradation in the rumen (Reprinted with permission from [38]).

In the barn floor, urinary urea-N (UNN) is hydrolysed to $\text{NH}_3/\text{NH}_4^+$ by urease enzyme, a nickel-dependent metalloenzyme found in a wide variety of environments, including animal faeces and floor surfaces [40, 41]. The urease activity depends on temperature, pH, UNN concentration, and floor characteristics (Figure 3). Neutral and basic pH favors NH_3 emission while acid pH favors NH_4^+ production [35, 42]. Additionally, organic N can be converted into NH_4^+ through mineralization, and consequently converted to NH_3 . In both processes, NH_3 volatilization is closely related to temperature, and ventilation rate [43]. Moreover, during the nitrification process, NH_4^+ is converted to

NO_3^- , which can be reduced to N_2 through denitrification, with nitric oxide and N_2O formed as intermediate products (Figure 3) [44]. Nitrous oxide can also be formed to a lesser extent, as an intermediate product in the rumen during fermentation of dietary proteins [26].

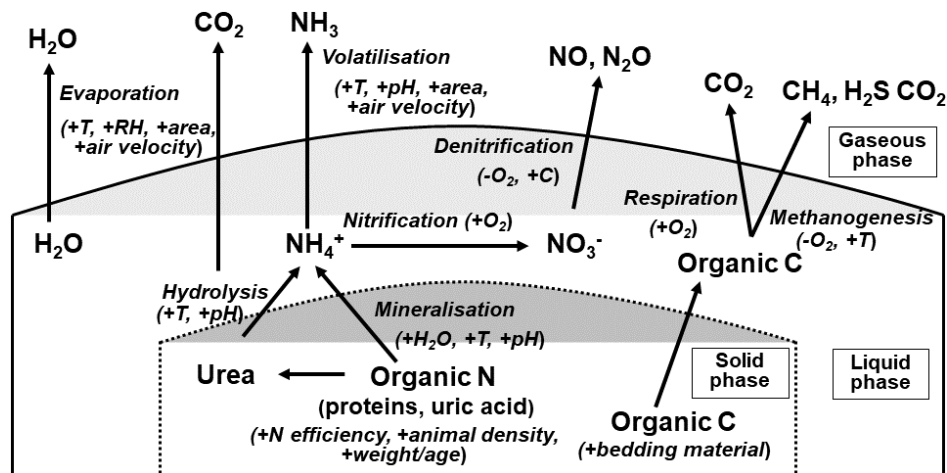


Figure 3 - Decomposition of the animal manure and main factors controlling carbon and nitrogen gaseous emissions (reprinted with permission from [46])

Enteric CH₄ emissions

In ruminant animals, the majority of CH₄ is produced in the rumen by methanogenic archaea, with a smaller amount generated in the large intestine through the methanogenesis process [46]. Briefly, in the forestomachs, plant structural carbohydrates are broken down by a consortium of microorganisms into volatile fatty acids (VFA), CO₂, and hydrogen (H₂) [47]. Ruminal VFA absorbed through the rumen wall (mainly acetic, propionic, and butyric acids) serves as the primary energy source for the animal. Among other functions, acetic acid is the primary precursor of milk fat [48]; propionic acid is the principal neoglucogenic nutrient [49]; and butyric acid is the main energy source for the ruminal epithelium [50].

Dissolved H₂ plays an important role in ruminal fermentation patterns and methanogenesis modulation. When H₂ concentration is low in the rumen, the production of acetate and butyrate increases, leading to higher CH₄ production; In contrast, a higher H₂ concentration favours the propionate production, competing with CH₄ for available H₂ [51].

In the rumen, CH₄ is primarily produced through hydrogenotrophic and methylotrophic pathways, with a smaller contribution from the acetoclastic pathway [52]. These pathways differ in the substrate that methanogenic archaea used for growing. However, the last step of methanogenesis is similar all three pathways, as CH₄ is catalysed by methyl-coenzyme M reductase (MCR, Figure 4). The methyl-coenzyme M reductase is a nickel enzyme bound in coenzyme F₄₃₀, and it is active in the 1+ oxidation state as Ni(I). This enzyme catalyses the reduction of methyl-coenzyme M (CoM) by coenzyme B, resulting in the production of CH₄ and the heterodisulfide of coenzyme M and coenzyme B (CoM-S-S-CoB, Figure 4) [53]. In the hydrogenotrophic pathway, the most predominant route for methanogenesis, H₂ is oxidized to H⁺ reducing CO₂ into CH₄, carried out by *Methanobrevibacter*, *Methanosphaera*, *Methanimicrococcus*, and *Methanobacterium* species [47, 54]. In the methylotrophic pathway *Methanosarcinales*, *Methanosphaera*, and *Methanomassiliicoccaceae* species use methyl compounds as substrate, while the acetoclastic pathway is carried out by *Methanosarcinales* species using acetate as substrate (Figure 4) [55, 56]. Although methanogenesis ensures the low pressure of H₂ in the rumen, CH₄ production represents an energy loss of feed energy for the animal (2% to 12%) in addition to environmental challenges [57, 58].

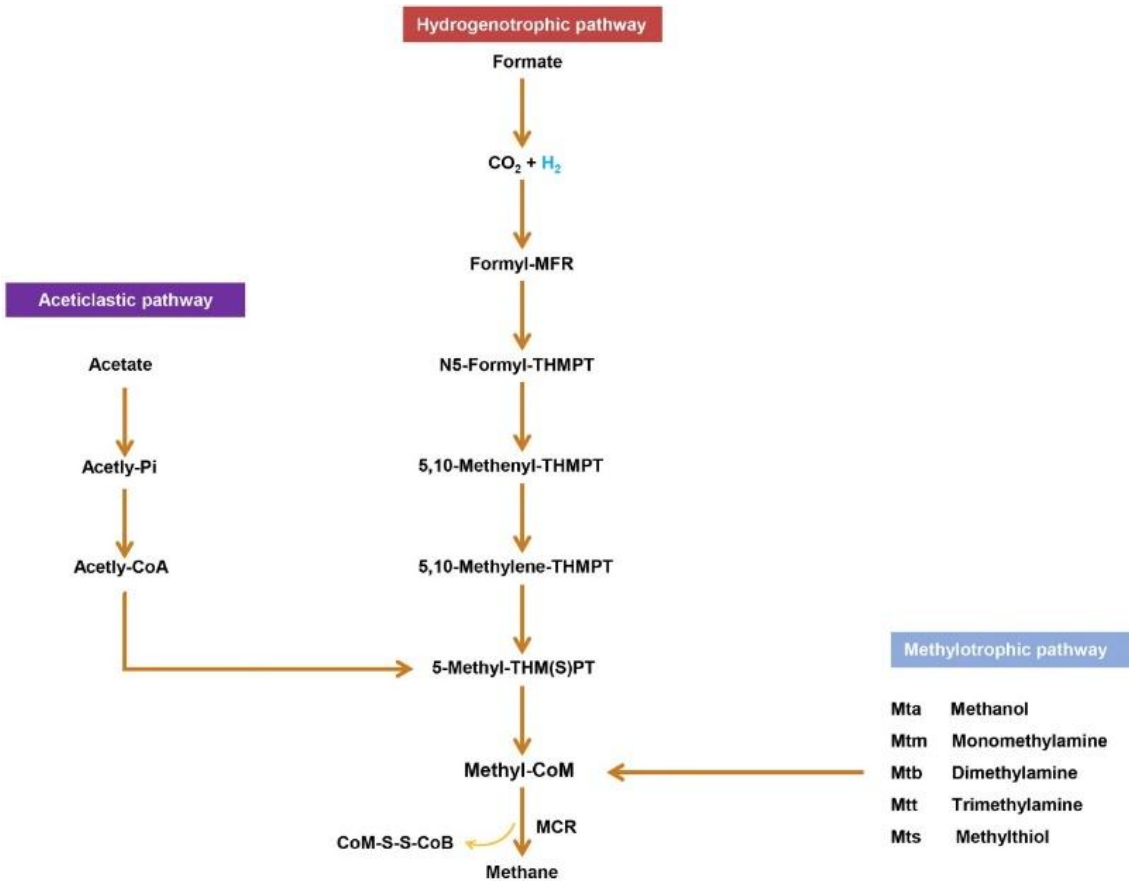


Figure 4 – CH₄ production through hydrogenotrophic, acetoclastic, and methylotrophic pathways. (Reprinted with permission from [52]).

Mitigation strategies for N emissions in dairy cattle barns

Approaches for N emissions reduction at the barn level include animals diet manipulation, ensuring appropriate manure management and adjustment of barn conditions and architecture [44, 60, 61]

Improving the prediction of metabolizable protein supply for dairy cows and shifting from urine to faecal N excretion are key strategies for improving N use efficiency and reducing N emissions [61, 62]. Huhtanen and Ahvenjärvi [61] demonstrated the potential for improving predictions of metabolizable protein supply in cow diets. This work underlines the overestimation of rumen undegradable protein compared to microbial protein, associated with an underestimation of both microbial mass protein and the contribution of protozoa to the overall protein flow. A suggested decrease of 1% in the protein content of animal feed could lead a reduction of up to 15% in total NH_3 emissions from barns, provided that a balanced supply of amino acids is maintained [63]. Precision feeding combined with accurate prediction of protein requirements can be used to reduce N excretion [61, 64]. Further research is needed to understand the correlation between efficiency on N utilization, N excretion, and N emissions from manure among different animals within the dairy herd, considering factors such as stages of lactation [65] and parity [66].

Appropriate manure management is effective in reducing N emissions in dairy cattle barn, especially of NH_3 . A cleaning system ensuring a dry floor and rapid removal of manure [67], a grooved floor system with perforations [68], or a double-sloped solid floor with a central gutter for urine drainage [69] can reduce NH_3 emission from the animal house by 26%, 46% and 50%, respectively. Several other barn conditions improvements can help manage temperature, ventilation rate, and relative humidity to reduce NH_3 emission potential [71]. Besides, additional abatement strategies for NH_3 are described in the work of Fangueiro *et al.* [60].

Slurry acidification [70-72], and urease inhibitors [73] applied to concrete floor surfaces are also effective approaches for NH_3 emissions abatement. The primary concern with handling strong acids is the significant hazard they pose which is the major disadvantage of slurry acidification. Additionally, the most common acids used present certain limitations. For example, organic acids degraded quickly and require large quantities to achieve the desired pH level. Nitric acid can lead to increase of N_2O emissions and N content of slurry, while sulphuric and phosphoric acids enhance the nutrient content of slurry, resulting in an excess of sulphur (S) and P [59, 63].

Mitigation strategies for enteric CH₄ emissions

Selective breeding, vaccines, dietary interventions, or methanogenesis inhibitors are promising strategies to reduce enteric CH₄ production. Table 1 summarizes the main mitigation opportunities for CH₄ reduction. The reduction effectiveness of mitigation strategies can be evaluated using different metrics, including CH₄ production (absolute emissions, g/d), CH₄ yield (g/kg of DMI), or CH₄ intensity (g/kg of meat, milk, or wool produced) [16, 74-77]. Breeding for feed efficiency and low-CH₄-emitting animals is the main challenge for researchers nowadays to enhance the sustainability of dairy cattle. There's a clear opportunity to develop strategies to increase feed efficiency and reducing CH₄ production, while improving knowledge on the relationship between both parameters. In fact, feed efficiency and CH₄ production are known to be a heritable trait and correlated to the ruminal microbiome [78, 79]. Currently, breeding goals focus on the following objectives: i) developing traits related to feed intake and GHG emissions ii) evaluating the relation between CH₄ production with other important economic traits, and iii) accurately measuring enteric CH₄ to estimate genetic parameters and differences among animals throughout the lactation cycle and across herds. These measurements will be incorporated into dairy selection indices of national breeding programs [80-82]. Additionally, this mitigation strategy is not expected to produce any upstream or downstream GHG emissions reorganization [75].

Dietary interventions can effectively decrease enteric CH₄ production (Table 1). These interventions include providing forages that are of high quality and digestibility [74], increasing the level of feed intake [16, 46], reducing the forage-to-concentrate ratio [51], providing tanniferous forages [83] incorporating dietary oils and fats [84, 85], oilseeds [86], *Asparagopsis* spp. [87], biochar [88], and the feed additive 3-nitrooxypropanol (3-NOP) [89]. Despite the differences in mode of action among these feed ingredients, the length of their effectiveness remains a significant challenge for their use, primarily due to the ruminal microbial resistance development [90]. These will be addressed in more detail below (Table 1).

Table 1. Strategies to reduce enteric CH₄ emissions.

Strategy	Mode of action	Reduction potential	Side effects	Limitations
Breeding	Selecting for animals with low CH ₄ emissions as CH ₄ emission is a heritable trait. Genetic progress is cumulative and permanent ^[91]	11 to 26% in 10 years are theoretically possible ^[92]	Unknown	Requires a large number of animals with records to predict accurate values. Scarce CH ₄ emissions data; measures are expensive and labor intensive ^[91] .
Increasing feeding level	Changes in rumen fermentation patterns inhibit the activity of methanogens ^[16]	↓CH ₄ I _M (9 to 23%) ^[16]	↓FD (2 to 12%), ↑WG (38 to 398%) and MY (10 to 25%) ^[16]	The potential impact of this practice on manure CH ₄ emissions, due to reduced fiber digestibility, needs to be assessed.
Decreasing grass maturity		↓CH ₄ I _M (7 to 18%) ^[16]	=FI ↑MY (1 to 18%) and ↑FD (9 to 21%) potentially leads to manure CH ₄ increase	Increased protein content can lead to higher N intake. Further investigation into the trade-offs related to manure N ₂ O emissions.
Decreasing dietary forage to concentrate ratio	Stimulate propionate fermentation in the rumen reducing the amount of H ₂ available for methanogenesis ^[85] Changes in rumen fermentation patterns inhibit the activity of methanogens ^[16]	↓CH ₄ I _M (4 to 14%) and CH ₄ I _G (3 to 15%) =absolute CH ₄	↑FI (5 to 14%), MY (10 to 24%) and WG (13 to 29%) =FD ^[16]	Feed availability, cost, risk of rumen function disturbances.
Tanniferous forages	Reduce fiber fermentation in the rumen to lower hydrogen and acetate production, inhibiting methanogenic microorganism growth ^[93]	↓CH ₄ I _M (8 to 26%)	↓FD (2 to 12%) which potentially leads to ↑manure CH ₄	More research is needed to confirm effects on overall animal performance. Decrease palatability and FI ^[16] . Can bind dietary protein, decreasing protein digestion and animal production ^[16] .

Table 1 (cont.). Strategies to reduce enteric CH₄ emissions.

Strategy	Mode of action	Reduction potential	Side effects	Limitations
Dietary oil and fat	Dietary oils and fats reduce H ₂ accumulation through fatty acid biohydrogenation, reducing the intake of fermentable OM, fiber digestion, and inhibiting methanogens	↓CH ₄ l _M (6 to 18%) and CH ₄ l _g (8 to 35%)	↓FD (2 to 17%) which potentially leads to manure CH ₄ increase and ↓FI (3 to 8%) =WG	Reduced DMI associated with negative effects on fibre digestibility in the rumen ^[85] . Reduction of CH ₄ production dependent on the relative proportion of forage and concentrates in the diet. Feeding diets too high in starches, oil and fats can have adverse effects on animal health and yields.
Oilseeds (Lactating animals only)	The use of oilseeds, due to its unsaturated fatty acids, could provide a H ₂ sink, inhibit methanogens as well as reduce fiber digestion and therefore CH ₄ production	↓CH ₄ l _M (4 to 19%) ^[74] ↓CH ₄ l _M of 8 % for an additional 20 g FA/kg dietary DM or 7 % for 20 g extra crude fat/kg dietary DM ^[85]	↓FD (6 to 11%) tended to ↓FI (1 to 17%) =MY	Variation in milk fat responses ^[85] . Oilseed supplementation is promising when mixed into the basal ration to provide a gradual daily supply of adequate doses of lipids ^[85] .
3-NOP	Oxidize the active site Ni(I) of methyl-coenzyme M reductase which inhibits the final catalytic step of methanogenesis	↓daily and yield CH ₄ by 28% ^[74]	=FI, FD, MY ^[74]	Potential decrease in the efficacy over time should be confirmed in future studies ^[74] .
<i>Asparagopsis</i> spp.	High content of halogenated analog compounds, such as bromoform, can inhibit the last step of the methanogenesis in the rumen	↓ yield CH ₄ (65 to 98%) and daily (55%)	↓ Total gas production, ↓DMI and ↓MY ^[94]	Reduced voluntary feed intake and potential damage to the rumen wall. Transfer of metabolites such as bromoform to milk, faeces, and urine ^[87] Limits of 5 mg/kg diet of iodine for lactating cattle can limit its use ^[95] .
Biochar	Provides gas adsorption Has a direct toxic effect on methanogens. Provides habitat for methanogenic-methanotrophic interactions	Yet to be proven experimentally	Yet to be proven experimentally	Contrasting results on CH ₄ production. Scarce information to establish a clear link between biochar characteristics, the level of inclusion, and their effects on CH ₄ production.

CH₄l_M – CH₄ emissions per unit of milk, FD – Fiber digestibility, DMI – Dry matter intake, WG – Weight gain, MY – Milk yield, FI- Feed, intake, CH₄l_g – CH₄ emissions per unit of gain.

Strategies of particular interest include 3-NOP, *Asparagopsis* spp., and biochar. The methanogenesis inhibitor 3-NOP, is patented since 2012, and requires regulatory approval for its use as a feed additive for enteric CH₄ mitigation in certain countries [16, 96]. Its structure is analogous to CoM, allowing it to bind to the active site of methyl-coenzyme M reductase, which inhibits the final catalytic step of methanogenesis (Figure 4, [97]). The reduction of CH₄ with 3-NOP supplementation shows a dose-response correlation, indicating higher efficiency in dairy compared to beef cattle [98-100]. Studies also reveal that the inclusion of 3-NOP does not affect the fiber digestibility [101] or dry matter intake (DMI) of dairy cattle [102], nor does it impact the weight gain of beef cattle [100]. However, studies demonstrate that 3-NOP inclusion effect varies with feed diet, particularly the dietary neutral detergent fiber (NDF) content [(Table 1), 74, 99]. Thus, future research is needed to explore the effect of 3-NOP on DMI, rumen fermentation parameters, and rumen microbiome [102].

Microalgae and seaweed biomasses have been used for centuries as source of food and feed due to their rich content of proteins, carbohydrates, minerals, vitamins, and bioactive compounds [103, 104]. In recent years, there has been increasing interest in microalgae for their antimethanogenic properties. Studies show that incorporating microalgae with high omega-3 polyunsaturated fatty acids content into ruminant diets can significantly reduce CH₄ production [105, 106]. This effect is marked with red seaweeds' inclusion, which contain phlorotannins and bromoform, demonstrating a reduction potential of up to 99% [(Table 1), 94, 107]. Bromoform is the most abundant halogenated CH₄ compound and is particularly found in *Asparagopsis taxiformis* and *Asparagopsis armata*. This halogenated compound inhibits the last step of methanogenesis by reacting with vitamin B12 and inhibiting methyl-coenzyme M reductase, similar to the action described in 3-NOP mode of action [108, 109]. However, exposure of animals to bromoform over long periods has been linked to the development of liver and intestinal tumours [110]. Additionally, studies demonstrate that *Asparagopsis* spp. supplementation lowers DMI [111-113], and more research is needed to explore the inclusion level effect on animal productivity [16]. Moreover, it has been shown that *Asparagopsis* spp. inclusion is responsible for iodine and bromide concentrations in milk [(Table 1), 94] and meat [113], which is hazardous for human health [87, 114].

Biochar plays a crucial role in circular agriculture models, which explains the ongoing interest in its use as a feed additive for enteric CH₄ mitigation (Table 1) [88]. It is a stable carbon-rich material produced through the pyrolysis of biomass under oxygen-limited conditions [115]. Due to its high porosity, and large surface area with high adsorption capacity biochar serves as a versatile additive for various applications, including in soil

amendment [116], water treatment [117], and GHG mitigation from soils [118], and during manure storage [119].

The use of biochar as a feed additive for enteric CH₄ reduction still faces several challenges (Table 1). The wide range of biomass sources and production parameters used influences biochar physicochemical properties, leading to biochar with different functional characteristics and applications [31]. Therefore, specific parameters such as elemental composition, surface chemical composition, structure, and stability vary among biochar materials [120, 121].

However, existing *in vitro* and *in vivo* studies on the topic show inconsistent results and fail to establish a clear link between biochar characteristics (such as biomass and temperature of pyrolysis), the level of inclusion, and their effects on CH₄ production [88]. For instance, in the *in vitro* short-term studies CH₄ production decreased at both high (700-1000 °C) and low (300 °C) pyrolysis temperature [122-128]. Conversely, at pyrolysis temperatures ranging from 350-700 °C either increased or was unaffected [129-131]. Regarding long-term incubations, a reduction in CH₄ production was observed only with acidic (pH 4.8) jack pine biochar, raising the importance of biochar pH on methanogenesis mitigation [132, 133]. Various modification methods have been studied to improve biochar properties, such as acidification of surface area, with inconsistent results [129, 132, 134, 135]. The uncertainty of biochar's effect on methanogenesis is also identified in the *in vivo* studies [125, 136-139]. Results suggest that using alternative electron sinks might enhance the symbiotic associations of biofilm on feed particles [125]. Besides, further research is needed to evaluate the effect of dietary biochar supplementation on microbial communities in both the rumen and manure [136, 140]. Approaches to reduce enteric CH₄ can result in GHG swapping emissions, which have been considered in the environmental impacts assessment of dairy farms as proxy for all production system (Figure 5) [141]. The potential of biochar as a cascade strategy for GHG mitigation should also be explored, due to the numerous applications of this porous material [119, 142-144].

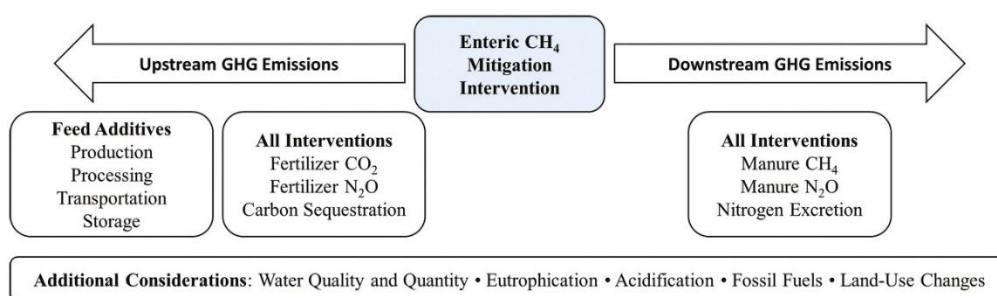


Figure 5 - Possible upstream and downstream effects of enteric CH₄ mitigation interventions (Reprinted with permission from [143]).

Aims and thesis outline

Desperate and effective measures are needed to reduce enteric CH₄ produced in the rumen and GHG emissions from manure decomposition, which is crucial for achieving compliance with the European directives and meeting the Portuguese environmental targets. In line with these environmental aims, on-field studies to assess gaseous GHG emissions from dairy farms will improve the emissions factors used in the national inventories on GHG emissions, allowing the adoption of accurate mitigation plans, and lowering the environmental impact from the agricultural sector.

This PhD thesis aims to evaluate a nutritional strategy to mitigate enteric CH₄ in dairy cattle and to assess GHG and NH₃ emissions of dairy cattle barns, contributing to fill the gap in knowledge regarding seasonal and daily gaseous emissions variations of Portuguese dairy farms.

To achieve these goals, three specific objectives were defined:

1. To explore the effect of biochar as feed additive to reduce CH₄ production from enteric fermentation.
2. To assess gaseous emissions potential of dairy cow excreta, in a simulated barn floor, from varying feeding systems, lactation and sampling times.
3. To identify seasonal and daily variations in CH₄ and NH₃ emissions in naturally ventilated dairy cattle barns through on-farm measurements.

This thesis is structured into six chapters. After the present General Introduction (Chapter One), Chapters Two to Five consist of peer-reviewed scientific articles, each corresponding to a published study.

Chapter Two overviewed the most relevant *in vitro* and *in vivo* assays in the literature evaluating biochar as a mitigation strategy for enteric CH₄, as well as its effect on rumen fermentation were reviewed in. Additionally, this chapter explores the potential impact of biochar on CH₄ emissions from manure.

Chapter Three assessed, for the first time, the effect of potato peel and agro-forestry biochar on CH₄ production and fermentation parameters, *in vitro* (24-h batch) incubations. Biochar's were incubated at two levels (0, 5 and 10%) with different substrates (maize silage and haylage).

Chapter Four explore the effect of i) feeding system; ii) stage of lactation; and iii) sampling times on CH₄, NH₃, N₂O, and CO₂ emission potential from dairy cows' excreta, in a laboratory chambers system simulating a solid concrete cattle house floor, for 48-h.

Chapter Five monitored CH₄ for the first time, and NH₃ emissions, for the second in naturally ventilated dairy cattle barns from Portugal. Measurements were performed in three farms from NW of Portugal, in the four seasons of two-years.

The final chapter, **Chapter Six**, presents the general discussion, overall conclusions and future perspectives, providing recommendations based on the findings of this PhD research.

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Chapter Two

Review: Heads or tails? Towards a clear role of biochar as a feed additive on ruminant's methanogenesis

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Abstract

The use of biochar has been suggested as a promising strategy in bio-waste management and greenhouse gases mitigation. Additionally, its use, as a feed additive, in ruminants has been reported to have contrasting effects on enteric CH₄ production. Hence, this chapter intends to overview the most relevant literature that exploited the use of biochar as a mitigation strategy for CH₄. This includes the reported effects of biochar on CH₄ production and rumen fermentation observed in *in vitro* and *in vivo* assays, as well as manure's CH₄ emission. The information available about the biochar and the experimental conditions used in the different studies is still limited, which created additional challenges in identifying the biological mechanisms that potentially drive the contrasting results obtained. Nevertheless, it is clear from the current state-of-the-art that biochar may be a key player in the modulation of gut fermentation and in the reduction of greenhouse gases produced by ruminants that need to be consolidated by further research.

Keywords: Biomass, Biochar, Enteric Methane, *In vitro*, *In vivo*, Ruminants.

Introduction

The livestock sector was estimated to emit 14.5% of global anthropogenic GHG, mainly CH₄, N₂O, and CO₂ [1], with enteric CH₄ corresponding to 40% of total livestock sector emissions, 77% of which emitted by cattle [1].

Ruminants are herbivorous animals that host a complex symbiotic microbial population composed of bacteria, protozoa, archaea, fungi, and bacteriophages in the two forestomach (reticulum and rumen) where feeds undergo fermentation, before entering the true stomach, the abomasum. Microbial population ferments structural and non-structural polysaccharides, and proteins originating VFA (mainly acetate, propionate, and butyrate), ammonia-N (NH₃-N), CO₂, and H₂ [2]. Volatile fatty acids are absorbed through the rumen wall and comprise the major energy source of the host animal. Hydrogen is mainly eliminated by the reduction of CO₂ by methanogenic archaea [3]. Methane is the second GHG contributor to climate change, with a global warming potential 28 times larger than CO₂, in a time horizon of 100 years, and enteric CH₄ represents a loss from 2 to 12% of total gross energy intake [4]. Mitigation of enteric CH₄ emissions is thus important not only to minimize the environmental impact of ruminant production but also to improve feed efficiency.

Several strategies have been evaluated to reduce enteric CH₄ production, including feeding management (e.g., ingredient selection, feed supplements, rate of passage, and better-quality ingredients), rumen modifiers (e.g., defaunation, bacteriocins, and immunization), and improvement of animal production through genetics (e.g., nutrient utilization, feed efficiency, and CH₄ production) [5], but effects are often transient [6] or conflicting [7]. Greenhouse gases (CH₄, N₂O) and ammonia (NH₃) are also produced during cattle manure decomposition in housing, storage, and treatment, and ultimately during land spreading [8]. Different strategies have been proposed to reduce gaseous emissions in each stage of manure management, from dietary manipulation to chemical application in slurry [9, 10]. One emerging strategy to cope with the mitigation of both enteric CH₄ and GHG from ruminants' manure is the use of biochar. Biochar is a stable porous carbon-rich material (between 65 and 90%), mainly produced by the pyrolysis method under oxygen-limited conditions, containing mineral elements whose physical and chemical characteristics are determined by feedstocks and technologies involved in the production process [11, 12]. Due to its characteristics, biochar has been studied for multiple uses, such as soil amending [13, 14, 15], mitigating GHG emissions from soil [16, 17, 18, 19], recovering nutrients from wastewaters [20], and reducing GHG emissions from cattle manure during storage [21, 22]. Its porous structure promotes soil moisture retention, reduces bulk density, enhances the OM content, and can positively affect soil cation

exchange capacity [23, 24]. Due to these properties, interest has emerged in biochar as a feed supplement to mitigate enteric and fecal CH₄, and manure gaseous emissions [25, 26], in a cascade approach, thus enhancing its effect along the cattle production system [27]. In this context, the European biochar foundation has developed guidelines for biochar production to be used as a feed additive [28] under the requirements of the European Food Safety Authority (EFSA) and respecting the commission regulation (EC) 178/2002 [29] and 834/2007 [30].

The role of biomass and production conditions on biochar characteristics

The biomass source and the type and conditions of production are key factors in biochar physicochemical properties resulting in different functional characteristics and applications [31], being pyrolysis the most common process for the production of biochar. The characteristics of biochar can be highly variable, especially in terms of elemental composition, surface chemical composition, structure, and stability. Each component's decomposition and depolymerization occurs through several reactions at different temperatures, contributing to the structural differences among biochars [32, 33].

In the works reviewed here, biochars were mainly produced by the pyrolysis of agriculture and forestry lignocellulosic biomasses, which are primarily composed of cellulose (40–45%), hemicellulose (25–35%) and lignin (20–30%), although their distribution varies among biomasses [34]. In terms of gaseous capture, the most relevant characteristics of biochar are the OM content (given by polarity and aromaticity), mineral content, cation exchange capacity, surface charge, and textural properties (surface area and pore size) [35].

The adsorption capacity of biochar related to the polarity and aromaticity is highly modulated by the pyrolysis conditions [35]. Due to the high carbon content and porous structure, adsorption is a valuable property of biochar, which has been used for environmental purposes, such as the reduction of GHG levels [35]. Therefore, the physical-chemical characteristics of the biochar have a strong influence on the capabilities of the materials for a particular application (Figure 6).

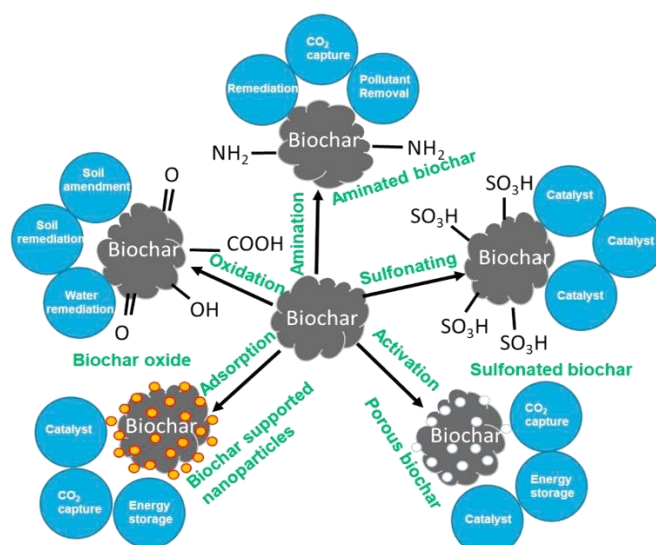


Figure 6 - Biochar post-production functionalization and potential applications. Reprinted with permission from [33].

For example, the ash content that results from the decomposition of the inorganic matter of biomass [23] is expected to be low in wood-based biomass when compared to mineral-rich biomass, such as grass, manure, litter, and solid waste [36]. Wood, bamboo, corncob, corn stover, pellets (miscanthus, softwood, wheat straw, and oilseed rape straw), rice straw, and potato peel biochar reported less than 25% of ash content, while rice husk presented higher than 40% [37, 38, 39, 40, 41, 42, 43, 44]. The ash content has been demonstrated to be relevant for the surface polarity and distribution of pores, thus influencing the sorption capacity of the material. The mineral content in biochar (such as carbonates, oxides, phosphates, alkali, or alkaline earth metals) has been shown to increase the sorption capacity for acidic gases, such as sulfur dioxide, hydrogen sulfide, and CO₂ [12].

The surface area and pore size can also be modified by chemical and physical activation following the carbonization process [45]. The modification of biochar with a CO₂-NH₃ mixture resulted in a surface area increase besides improving the chemical properties of the surface by a nitrogen modification [46]. The microporous structure has a key role in CO₂ capture at low temperatures [47].

Using lignocellulosic biomasses (the main raw material present in the application herein described), a microporous structure is expected with higher cellulose and hemicellulose content, whereas mesoporous structures are expected with higher lignin content [27]. The increase in pyrolysis temperatures also increases the porosity, surface area, pH, ash, and carbon content of biochar due to the release of volatile components, while reducing biochar exchange capacity and yield [20, 48]. In the study of Calvelo Pereira *et al.* [38], an increase in surface area, carbon, nitrogen, and ash contents of biochar

produced from the pyrolysis of pine chips and corn stover was observed. Also, biomass has been shown to highly influence the surface area, as demonstrated by other authors [40, 44].

Effects of biochar on *in vitro* rumen fermentation

There is a paucity of data on the effects of biochar on CH₄ production by short- and long-term *in vitro* studies. Therefore, these will be addressed separately.

In vitro short-term studies

Table 2 presents the results obtained in 14 studies evaluating the effects of biochar addition up to 16% on rumen fermentation and CH₄ production through *in vitro* short-term incubations (up to 48 h). No clear association is evident between effects on CH₄ production and biochar characteristics (e.g., biomass, temperature of pyrolysis) and level of inclusion. Increasing pyrolysis temperature increases surface area, which has the potential to improve biofilm formation and promote the adsorption capacity of microorganisms, nutrients, and gases, thus reducing CH₄ production [26, 49]. Indeed, some studies [44, 50, 51, 52, 53, 54] reported a decrease in CH₄ production with the addition of biochar produced at very high temperatures (700–1000°C), whereas in the studies using biochar produced at lower temperatures (350–700°C) no effect [38, 55] or an increased [42] CH₄ production was observed. However, Saenab *et al.* [56] reported a decrease in CH₄ production when biochar from cashew nutshell was produced at 300°C and Cabeza *et al.* [40] found higher CH₄ production with biochar produced at 700°C than 550°C. It must be realized that *in vitro* systems do not effectively reproduce the *in vivo* situation, particularly the adaptation of rumen microbiome to novel materials, and for this reason, effects *in vitro* might not be observed *in vivo* [5].

The information about biochar characteristics (besides pyrolysis temperature), is absent in the majority of the studies, making impossible any association between the results and the biochar characteristics and their respective effects on CH₄ production. Despite not having evaluated the effect on CH₄ mitigation, McFarlane *et al.* [39] found biochar particle size to affect rumen fermentation, being inhibited with large particles (>178 µm vs. <178 µm). Although without impact on gas production and VFA proportions, these authors reported *in vitro* true digestibility of orchard grass hay to be increased by the inclusion of fine biochar particle size [39].

Table 2. Biochar biomass, temperature of pyrolysis (°C), and inclusion level (% dry matter basis) effects on CH₄ production in short-term *in vitro* studies.

Biomass	Temperature	Time (h)	Incubation level	CH ₄ production	Reference
Rice husk	900-1000	24	0, 1, 2, 3, 4, 5	↓ with 1% biochar; no further benefits with 2-5% biochar	[51]
		24	0, 0.5, 1	↓ with 0.5 and 1%; further reductions with addition of nitrate N and urea	[51]
		24	1.5	↓ with adapted inoculum or biochar addition	[57]
	700-900	24,48	1	↓ with higher reduction at 48 h	[50]
	1000	24	1	↓	[53]
	1000	6, 12, 18, 24	1	↓ at 18-24h	[54]
Pine wood chips	350	2, 6, 12, 24	16	Not affected	[38]
	550				
Corn stover	350				
	550				
Gasified					
Straw-based					
Wood-based	-	48	9	Not affected	[37]
Activated carbon					
Miscanthus straw pellets	550	24	1	↓ with biochar over the control	[40]
	700		10	No differences between sources ↑ with 700°C over 550°C	

Table 2 (cont.). Biochar biomass, temperature of pyrolysis (°C), and inclusion level (% dry matter basis) effects on CH₄ production in short-term *in vitro* studies.

Biomass	Temperature	Time (h)	Incubation level	CH ₄ production	Reference
Oil seed rape straw pellets	550	24	1	↓ with biochar over the control No differences between sources ↑ with 700°C over 550°C	[40]
	700		10		
Rice husk	550		1		
	700		10		
Cashew nutshell	300	3, 6, 9, 12, 24, 30, 36, 48	0, 0.75, 1.5, 2.25, 3	↓ with biochar ↓↓ with biochar and biofat	[56]
Potato peel	500	24	0, 5, 10	↑ over the control	[42]
Agro-forestry	600	24			
Mixed species of green waste tree pruning	500	6, 12, 24	0, 0.5, 1, 2, 4	Not affected by inclusion level	[55]
Rice straw	300, 500, 700	4, 24, 48	3	↓ with rice straw and corncob in comparison to bamboo at 4 and 48 h ↓ with increasing temperature	[44]
Corncob					
Bamboo					

A comparison between studies is further complicated by the diversity of biomass sources used (e.g., rice husk, pine wood, corn stover, cashew nutshell, tree pruning, rice straw, corncob, bamboo) that might affect VFA profile, thus introducing a confounding effect on the mechanism of CH₄ reduction. Most studies that compared the impact of biomass sources on enteric CH₄ production [37, 38, 41, 42] observed no differences among biochar sources. Conversely, Van Dung *et al.* [44] found rice straw and bamboo biomass to reduce CH₄ production compared to corncob, at 4 and 48 h of incubation, but not at 24 h. Moreover, these authors observed an interaction effect between biomass source and pyrolysis temperature [44], supporting the need for a multi-aspect analysis of biochar's chemical and physical properties. The effects on VFA profile were further assessed [38, 40, 42, 55, 56]. In the study of Calvelo Pereira *et al.* [38], despite a decrease in propionate proportion found with some mixtures, which might indicate an increase in H₂ produced, the effects were insufficient to affect CH₄ production. In the study by Cabeza *et al.* [40], the addition of biochar slightly reduced CH₄ production, but it kept unchanged the amounts of total VFA or acetate produced and reduced those of propionate and butyrate. Saenab *et al.* [56] observed a reduction of CH₄ production by 11.5% with 3% [dry matter (DM) basis] cashew nutshell biochar supplementation, although total and individual VFA produced were unaffected. Rodrigues *et al.* [42] attributed the reduction of VFA production through biochar addition to a reduced energy supply for microbial growth. Supplementation of tree pruning biochar up to 4% (DM basis) did not affect CH₄ or VFA content and profile [55].

The study by Leng *et al.* [57] was the only one that evaluated the effect of rumen fluid adapted to biochar. The authors attributed the reduction in CH₄ production with rumen-adapted inoculum to a larger ruminal population that oxidizes CH₄. Indeed, adapted rumen inoculum is expected to present a higher density of methanotrophs [58], possible the effect of biochar on rumen CH₄ is solely due to the increase in potential habitat for this consortium. However, in the study by Leng *et al.* [57], CH₄ reduction was higher with biochar addition to unadapted rumen inoculum than without biochar addition to adapted rumen inoculum. Biochar addition promotes either the association of microorganisms that more efficiently ferment feed materials or facilitates CH₄ oxidation by bringing together methanogenic archaea and methanotrophic consortia [59].

However, from the available studies, the mechanism of CH₄ reduction through biochar is unclear. Although biochar favors methanotrophism in the soil [60], the anaerobic rumen precludes the growth of aerobic methanotrophs, thus the action of biochar is most possibly through the promotion of micro-environments by the large surface area of biochar [40].

***In vitro* long-term studies**

The long-term effects of biochar supplementation on rumen fermentation and CH₄ production were further assessed *in vitro* using the rumen simulation technique system (Table 3). Despite differences among biochar biomass, pyrolysis temperature, and chemical and physical characteristics, only one study observed a CH₄ mitigation effect of biochar when compared to control [41]; supplementation levels (0.5, 1, and 2%, DM basis) having a quadratic effect, greatest with 0.5% inclusion. Jackpine biochar also improved most fermentation parameters (e.g., NH₃-N, total VFA, acetate, propionate, butyrate, and branched-chain VFA yield), nutrient digestibility (DM, crude protein, NDF, acid detergent fiber), and microbial N of total and liquid associated bacteria while decreased that of loosely associated bacteria [41]. Conversely, mineral-activated blackbutt [61], jack/yellow pine [43] and spruce stem [62] biochar supplementation kept unaffected gas production, fermentation parameters (pH, NH₃-N, total and individual VFA yield), nutrient digestibility, microbial N produced, protozoa count, and bacterial diversity, richness, and relative abundance. Inconsistency of biochar effects has been attributed to variations in biochar chemical and physical properties, including particle size, adsorptive potential, electrical conductivity, and electron-mediation in redox reactions [37, 39]. Several modification methods have been used to improve biochar properties, such as acidification of surface area, to increase biochar adsorption [23]. Teoh *et al.* [61] further suggested that biochar pH could be of particular importance in enteric CH₄ reduction, based on the notable CH₄ reduction (25%, as mg/g DM incubated) of the acidic (pH 4.8) jack pine biochar used in Saleem *et al.* [41] study. Acidic biochar has been associated with improved carbon sequestrum and higher redox potential in soils, whereas neutral mineral-rich biochar lacked this ability [63]. However, acidic (pH 4.9) pine biochar failed to reduce enteric CH₄ production [43] similarly to observed with basic (pH 8.2) biochar supplementation [37, 38, 61].

Acidic biochar has also been suggested to improve the redox potential and thus increase biofilm development by the mediation of electrons among the microbial population [61, 64]. However, more developed biofilms were observed on readily digestible substrates than on biochar surfaces [62, 65]. Even though microbial diversity, richness, and relative abundance were not affected by long-term biochar supplementation, discriminant analysis unveiled biochar-type specific changes in rumen bacterial families [43, 61, 62]. Of particular interest, Teoh *et al.* [61] found a 19.8-fold reduction in the abundance of Methanomethylophilaceae with the supplementation of mineral-activated biochar. Members of Methanomethylophilaceae family are methanogenic archaea that use sources of H₂ to reduce methylated compounds and produce CH₄ [66, 67], thus suggesting the potential mitigation effect of hardwood biochar [61].

Table 3. Biochar biomass, temperature of pyrolysis (°C), and inclusion levels (% dry matter basis) effects on rumen fermentation and CH₄ production in long-term *in vitro* studies.

Biomass	Temperature	Inclusion level	Substrate	Effects	Reference
Jack pine	600	0, 0.5, 1, 2	Barley silage: rolled barley grain: canola meal: concentrate (60:27:10:3)	Compared to control ↓ CH ₄ and ↑ VFA = gas, pH, protozoa Linearly ↑ N-NH ₃ , DMD, CPD, NDFD, ADFD, total and LAB microbial N	[41]
Hardwood blackbutt, clay and minerals	650	0, 3.6, 7.2	Oaten pasture: maize silage: concentrate (35:35:30)	= CH ₄ and total gas, pH, N-NH ₃ , VFA, DMD, microbial richness and diversity 7.2% tended to ↓ CH ₄ compared to 3.6%	[61]
Spruce steem	450	2	Barley silage: rolled barley grain: canola meal: premix (60:27:10:3)	Tended to ↓ CH ₄ (% total gas) = total gas, pH, VFA, protozoa, microbial N, bacterial richness, diversity and relative abundance	[62]
Jack-pine/ yellow pine	400-600	2	Barley silage: rolled barley grain: canola meal: premix (60:27:10:3)	= CH ₄ , total gas, pH, VFA, protozoa, microbial N, bacterial richness, diversity and relative abundance	[43]

NH₃-N- ammonia-N, DMD- dry matter digestibility, CPD- crude protein digestibility, NDFD- neutral detergent fiber digestibility, ADFD- acid detergent fiber digestibility, LAB- liquid associated bacteria, and VFA- volatile fatty acids.

Effects of biochar *in vivo*

The porous structure of biochar can adsorb gases and provide habitat for microbial biofilms [37, 68], which in addition to electron-mediation properties in biological redox reactions [69] suggest its potential to reduce enteric CH₄ production and promote rumen fermentation. As previously stated, *in vitro* studies present several advantages, but do not fully simulate the *in vivo* animal. Few studies have evaluated, *in vivo*, the effects of dietary biochar inclusion on ruminant performance and CH₄ production (Table 4). Globally, dietary supplementation with biochar from different sources increased or not affected ruminant performance and reduced or kept unaffected CH₄ production. Leng *et al.* [52] pointed out the need for CH₄ mitigation strategies to include alternative electron sinks rather than just focused on methanogens inhibition, due to the need for symbiotic associations in biofilm microbial colonies on feed particles for successful ruminal fermentation to occur. Rumen microbial biofilms are of particular importance for fiber fermentation, with microbial attachment to feed particles allowing pit formation as well as glycocalyx emission to fibrous amorphous material [75].

In Angus × Hereford heifers, Terry *et al.* [65] found that, although total tract digestibility, nitrogen balance, and CH₄ production were not affected by dietary biochar inclusion, the relative abundance of Fibrobacter and Tenericutes were reduced and that of Spirochaetaes, Verrucomicrobia, and Elusimicrobia increased. Modulation of the manure microbial population was also found to be affected by dietary biochar supplementation. Al-Azzawi *et al.* [74] reported decreased methanogenic population by 30% with a corresponding increase in the non-methanogenic archaeal species in manure, suggesting that formed CH₄ could be reduced by further utilization by methanotrophic species. Moreover, biochar was shown to affect nitrification by increasing ammonia-oxidizing organisms and reducing ammonooxygenase activity [76].

Table 4. Effect of biochar biomass and inclusion level (% dry matter basis) on ruminant performance and CH₄ production.

Animals	Diet	Biochar level (source)	Observations	Reference
Cattle (80-100 kg)	Cassava root chips and fresh cassava foliage	0.6 (Rice husks)	Live weight gain ↑ 25% ↑ DM feed conversion ↓ CH ₄ production	[52]
Angus × Hereford heifers (565 ± 35 kg)	Barley silage-based diet	0, 0.5, 1, 2 (Pine enhanced biochar)	CH ₄ emissions not affected Specific rumen microbiota altered	[65]

Table 4 (cont.). Effect of biochar biomass and inclusion level (% dry matter basis) on ruminant performance and CH₄ production.

Animals	Diet	Biochar level (source)	Observations	Reference
Crossbred steers (529 ± 16 kg)	Growing diet: brome hay: wheat straw: corn silage: wet distillers grains: supplement (21:20:30:22:7) Finishing diet: dry-rolled corn: corn silage: wet distillers: supplement (53:15:25:7)	0, 0.8, 3 (Whole pine trees)	CH ₄ tended to decrease in the growing animals CH ₄ not affected in the finishing animals	[70]
Lambs (37.9 ± 0.8 kg)	Alfalfa and barley (60:40) <i>ad libitum</i>	0, 2 (Lodgepole pine and quaking aspen)	= feed intake and average daily gain ↑ DM digestibility and digestible DM matter intake	[71]
Kermanian ram lambs (21.9 ± 2.24 kg)	Alfalfa: wheat straw: concentrate (30:10:60)	0, 1, 1.5 (Walnut shell and pistachio by-product at 1%, chicken manure at 1.5%)	= DM intake ↑ average daily gain ↑ feed conversion ratio	[72]
Bos taurus crossbred beef steers initial (286 ± 26 kg)	High-forage and high-grain diets	0, 0.5, 1, 2 (Yellow pine)	2% lean meat yield = body weight and DM intake	[73]
Milking dairy cows	Barley hay and compound (40:6) Free-access to forage during the day	0.5 (Powdered activated carbon)	↓ manure CH ₄ by 30–40% and CO ₂ emissions by 10% ↑ milk production ↓ manure methanogenic flora by 30% ↑ nonmethanogenic species	[74]

DM- dry matter, and CO₂- carbon dioxide.

Although dietary biochar supplementation had variable effects on ruminant performance, these were overall promising and suggest potential benefits beyond methanogenesis. Indeed, 0.6% biochar increased the live weight gain of yellow cattle and DM feed conversion by 25% [52]. Terry *et al.* [73] found no effect on body weight gain or DM intake in beef steers up to 2% biochar, but lean meat yield increased with the highest biochar level tested (2%). In lambs, 2% biochar gain-to-feed ratio and average daily gain unaffected, and improved DM intake [71], while up to 1.5% biochar was found to maintain DM intake and increase average daily gain and feed conversion ratio [72]. In addition, milk production of cows fed 0.5% (DM basis) activated carbon was improved [74]. Furthermore, in an innovative solution for biochar utilization reported by Joseph *et al.* [77], biochar was mixed with molasses and fed directly to cows, the dung-biochar mixture being incorporated into the soil profile by dung beetles and the costs and benefits of integrating biochar with

animal husbandry and improvement of pastures were assessed. These authors found that dung-biochar had an outer coating of mineral elements (P, K, Mg, Ca, Al, Si, and Fe) and nitrogen, adsorbed in the cow gut, that were available for soil, thus being an effective strategy to improve soil properties. In addition, increasing returns to farmers were calculated, suggesting the profitability of dietary biochar supplementation in ruminant production systems [77].

Notwithstanding, the inconsistent results in the literature on the effect of biochar on reducing CH₄ emissions, rumen *in vitro* fermentation, and *in vivo* rumen function limits the mechanistic understanding of the underlying mode of action. This is particularly difficult due to the use of different sources of biomass and production conditions, such as duration and temperature, of pyrolysis as well as post-treatment modifications, which alter the composition, porosity, and chemistry of biochar [65], but also to the poorly characterized biochar used in ruminant studies. These challenges make comparisons between studies difficult, and in addition to the lack of knowledge of the long-term effects of dietary biochar supplementation, could have limited its use in ruminant feeding practices on-farm.

Effects of biochar on manure CH₄ production

Ruminant production generates high amounts of manure that need to be stored until the land application. Manure is a rich source of nutrients, and its application is shown to improve soil quality, to reduce the use of mineral fertilizers and costs of production [21]. However, during manure storage and land application, malodorous compounds and GHG, such as CH₄, CO₂, and N₂O, as well as NH₃, are formed and emitted [78], with a detrimental impact on ecosystems [22]. Biochar application to manure can be an effective strategy to improve its environmental impact, as it can absorb and retain GHG, NH₃, and nutrients [79, 80]. Moreover, when applied to soils, biochar-enriched manure may provide nutrients, sequester carbon, and improve soil's structure [22, 79]. Although the already identified biochar potential in manure, differences have been reported among biochar biomass, production conditions, pH, hydrophobicity, and particle size [22, 68, 81]. Moreover, a life cycle assessment of the environmental implications of stored cattle slurry (a mixture of manure, split feed, and water) treatments revealed biochar to be one of the less effective approaches to suppress GHG emissions from liquid slurry, except for N₂O [21]. The inconsistent results from biochar application to manure pinpoint the need for more research in this field.

Conclusions

Biochar is undoubtedly a material with high potential to deal with ruminant methanogenesis due to its availability, stability, and large surface areas. Nevertheless, there is a significant knowledge gap about the mechanisms that govern the interactions between biochar and the plethora of microorganisms that are present in the ruminant's gut and manure. In this chapter, we addressed the most relevant literature on the topic, seeking additional clarification about the potential role of biochar in methanogenesis. The absence of detailed characterization of biochar used, and the diversity of the experimental conditions applied in the different studies, create additional challenges for a critical comparison of the past findings. Therefore, for future studies, some level of standardization and the detailed characterization of the biochar(s) used will have a significant impact on the clarification of its role in the mitigation of GHG emissions from ruminants.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Ana R.F. Rodrigues: Investigation; Writing - original draft; review & Editing. Margarida R.G. Maia: Conceptualization; Writing - review & editing. Ana R.J. Cabrita: Conceptualization; Writing - review & editing. Hugo M. Oliveira: Writing - review & editing. Inês M. Valente: Writing - review & editing. José L. Pereira: Supervision; Writing - review & Editing. Henrique Trindade: Supervision; Writing - review & Editing. António J.M. Fonseca: Conceptualization; Funding acquisition; Supervision; Writing - review & editing.

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Chapter Three

Assessment of potato peel and agro-forestry biochars supplementation on *in vitro* ruminal fermentation

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Abstract

The awareness of environmental and socio-economic impacts caused by greenhouse gas emissions from the livestock sector leverages the adoption of strategies to counteract it. Feed supplements can play an important role in the reduction of the main greenhouse gas produced by ruminants—CH₄. In this context, this study aims to assess the effect of two biochar sources and inclusion levels on rumen fermentation parameters *in vitro*.

Two sources of biochar (agro-forestry residues, AFB, and potato peel, PPB) were added at two levels (5 and 10%, dry matter (DM) basis) to two basal substrates (haylage and corn silage) and incubated 24-h with rumen inocula to assess the effects on CH₄ production and main rumen fermentation parameters *in vitro*.

AFB and PPB were obtained at different carbonization conditions resulting in different apparent surface areas, ash content, pH at the point of zero charge (pHpzc), and elemental analysis. Relative to control (0% biochar), biochar supplementation kept unaffected total gas production and yield (mL and mL/g DM, $p = 0.140$ and $p = 0.240$, respectively) and fermentation pH ($p = 0.666$), increased CH₄ production and yield (mL and mL/g DM, respectively, $p = 0.001$) and NH₃-N ($p = 0.040$), and decreased total VFA production ($p < 0.001$) and H₂ generated and consumed ($p \leq 0.001$). Biochar sources and inclusion levels had no negative effect on most of the fermentation parameters and efficiency. Acetic:propionic acid ratio ($p = 0.048$) and H₂ consumed ($p = 0.019$) were lower with AFB inclusion when compared to PPB. Biochar inclusion at 10% reduced H₂ consumed ($p < 0.001$) and tended to reduce total gas production ($p = 0.055$). Total VFA production ($p = 0.019$), acetic acid proportion ($p = 0.011$) and H₂ generated ($p = 0.048$) were the lowest with AFB supplemented at 10%, no differences being observed among the other treatments. The basal substrate affected most fermentation parameters independently of biochar source and level used.

Biochar supplementation increased NH₃-N content, *iso*-butyric, *iso*-valeric and valeric acid proportions, and decreased VFA production suggesting a reduced energy supply for microbial growth, higher proteolysis and deamination of substrate N, and a decrease of NH₃-N incorporation into microbial protein. No interaction was found between substrate and biochar source or level on any of the parameters measured. Although AFB and PPB had different textural and compositional characteristics, their effects on the rumen fermentation parameters were similar, the only observed effects being due to AFB included at 10%. Biochar supplementation promoted CH₄ production regardless of the source and inclusion level, suggesting that there may be other effects

beyond biomass and temperature of production of biochar, highlighting the need to consider other characteristics to better identify the mechanism by which biochar may influence CH₄ production.

Keywords Agro-forestry biochar, Supplementation level, Rumen fermentation, Potato peel biochar, Enteric methane.

Introduction

A multidisciplinary effort for developing efficient approaches for greenhouse gas emissions mitigation emerged from the growing concerns on global climate change. The livestock sector represents 14.5% of carbon dioxide equivalent of the total anthropogenic emissions [1], ruminants being estimated to be responsible for c.a. 80% of these emissions. In ruminant production, independently of the species, CH₄ comprises the largest source of greenhouse gas emissions, with enteric fermentation being responsible for 90% of CH₄ production and manure for the remaining 10% [2]. Enteric CH₄ production occurs by the reduction of carbon dioxide with H₂ by the rumen archaea population [3]. Methanogenesis not only contributes to the impact of ruminant production on climate change but also represents 2-12% of energy lost by the animal [4]. Hence, strategies to reduce CH₄ enteric production may lead to a win-win situation to the environment and economy of the livestock sector.

Biochar, a carbon-rich product obtained by thermal decomposition of biomass under a limited supply of oxygen at high temperatures (typically lower than 700 °C), has been successfully used to amend soils and to increase their nutrient availability beyond a fertilizer effect [5]. Due to its carbon sequestration ability [5], biochar can contribute to mitigate the emissions of CH₄ from soils [6], while promoting soil structure and crop production [7]. These results triggered a growing interest in the dietary supplementation of biochar as a potential strategy to reduce CH₄ formed during the rumen fermentation. The mechanism behind the decrease of gas and CH₄ productions through biochar supplementation is still unclear, but possible explanations include: (i) gas adsorption [8], (ii) a direct toxic effect on methanogens [9], (iii) substrate for the biofilms that provide a favorable habitat for the synergic methanogenic-methanotrophic interactions [9, 10], and (iv) electron mediators in biochemical redox reactions, enabling direct interspecies electron transfer [11, 12].

Furthermore, the oral administration of biochar to ruminant animals may have additional advantages, namely in the (1) sequestration of the GHG emitted from faeces and manure, in the (2) reduction of water and air pollution caused by the direct application of manure to soil [13], and in the (3) improvement of nutrient intake efficacy and of overall animal health [14]. Notwithstanding these facts, few studies have evaluated the effect of biochar on rumen fermentation. The current studies suggest that effects on rumen fermentation depend on biochar source and size [15], albeit effects on CH₄ are contradictory. Indeed, biochar supplementation was reported to reduce [16, 17] or kept unaffected [8, 17] enteric CH₄ production. Therefore, more research is needed to determine the role and potential benefits of different biochar sources and inclusion levels on rumen fermentation parameters. In addition, the putative interaction of biochar sources and inclusion levels with the basal

substrates should be examined. Indeed, Castro-Montoya *et al.* [18] reported interactions between CH₄ mitigation additives and basal substrates on CH₄ and VFA productions.

In this context, this study expands the knowledge about the role of biochar in rumen fermentation. It assesses, for the first time, the effect of agro-forestry (AFB) and potato peel (PPB) biochars, included at two inclusion levels (5, and 10% dry matter, DM, basis) on rumen fermentation *in vitro* (24-h batch) incubations. Basal substrate source (haylage, HS, and corn silage, CS) on biochar supplementation effects were also evaluated.

Materials and Methods

Effects of biochar supplementation on the rumen fermentation parameters were evaluated in 24-h *in vitro* incubations, according to a factorial design with two biochar sources (AFB and PPB), two biochar inclusion levels (5% or 10%, DM basis), and two basal substrates (HS and CS).

Biochars and basal substrates

AFB and PPB were produced from by-products of the Portuguese food industry and forestry sector.

AFB was produced through the slow pyrolysis of agro-forestry biomass wastes (vineyard pruning residues and acacia wood) at a temperature of 600 °C for c.a. 6 h. PPB was produced by lab-scale pyrolysis conducted in a quartz reactor, which was placed in a vertical furnace; potato peel waste from an appetizer industry was heated up to 500 °C at 10 °C/min, and the temperature held for 2 h under a N₂ flow of 150 mL/min. Conditions chose for AFB and PPB production were based on previous thermogravimetric analysis (TGA) and pyrolysis experiments that showed thermostability of these materials and therefore a reasonable biochar amount.

HS and CS were used as basal substrates in the *in vitro* incubations as well as dietary ingredients of the rumen inocula donor diets. Basal substrates were dried in a forced-air oven, for 48 h at 65 °C, ground to pass through a 1-mm screen and stored at room temperature until incubation.

Biochars characterization

The textural properties of biochars such as apparent surface area, total pore and micropore volumes were evaluated by the adsorption isotherm of N₂ at -196 °C obtained in an Accelerated Surface Area and Porosimetry System (ASAP 2010, Micromeritics

Instruments Corp. Norcross, GA). Adsorption measurements were made after outgassing the sample overnight under vacuum at 150 °C. The adsorption data were used to calculate the apparent surface area (S_{BET}) through the BET equation [19]. The total pore volume (V_{total}) was determined by the amount of N_2 adsorbed at the relative pressure $p/p_0 = 0.99$. The micropore volume (V_{micro}) was evaluated by the t-plot method, and the mesopore volume (V_{meso}) was determined by the difference between V_{total} and V_{micro} .

The surface chemistry of the biochars was characterized by the determination of the pH at the point of zero charge (pH_{PZC}), based on the following methodology: 0.1 g of biochar was mixed with 20 mL of 0.1 M NaCl solution in different flasks. The pH of NaCl solution was adjusted with the addition of 0.1 M solutions of NaOH or HCl to the desired initial pH value (between 2 and 12) in different flasks. Biochars were then added to each flask, the mixtures were shaken for 24 h to reach equilibrium, and the final pH measured (MicropH 2001 pH meter, Crison Instruments SA, Spain). The pH_{PZC} value corresponds to the plateau of the curve pH_{final} vs $\text{pH}_{\text{initial}}$.

Elemental analysis to determine C, H, N, and S contents in biochars was performed on an Elemental Analyzer Thermo Finnigan (CE Instruments, model Flash EA 1112 CHNS series) based on sample combustion dynamics. Ash content was also determined at 750 °C as specified in ASTM D 1762 standard (wood charcoal).

Rumen inocula donor animals and diets

Two adult Holstein cows, dry and not pregnant, fitted with rumen cannula (10 cm diameter; Bar Diamond Inc., Parma, ID), housed at the Vairão Agricultural Campus of School of Medicine and Biomedical Sciences, University of Porto (ICBAS-UP, Vila do Conde, Portugal), were used as rumen inocula donors. Care and management of cows followed the European Union good animal practices (Directive 2010/63/EU). Animal procedures and methodologies were reviewed and approved by the Animal Ethics Committee of ICBAS-UP, licensed by the Portuguese Directorate-General of Food and Veterinary Medicine (permit #FT2014DGV 046412 ICB), and conducted by trained scientists (FELASA category C).

To achieve true replication of the rumen inocula, cows were fed three total mixed rations (TMR) in three experimental periods of 30 days each. In the first period, one cow was fed a HS-based TMR and the other cow a CS-based TMR. Diets were exchanged between cows in the second period and, in the third period, one cow was fed a TMR based on CS and HS (Table 5). Cows were fed twice a day, at 09:30 h and 17:30 h, the daily amount of TMR being equally divided between meals. Orts were collected daily. Cows had *ad libitum* access to fresh water. After the 30 days adaptation of each experimental period, contents from all

quadrants of the rumen were collected to a pre-warmed (39 °C) thermos container and transported to the laboratory within 60 min.

Table 5. Ingredient and chemical composition (g/kg dry matter) of the animal diets and basal substrates used in the *in vitro* incubations

	Cows diets			Basal substrates	
	TMR1	TMR2	TMR3	Haylage	Corn silage
Ingredient composition, g/kg DM					
Haylage	723	-	491		
Corn silage	-	452	242		
Wheat straw	-	224	-		
Concentrate	277	324	267		
Chemical composition, g/kg DM					
Ash	80.1	56.6	69.4	72.7	34.5
Organic matter	920	943	931	927	965
Crude protein	104	116	119	55.3	77.4
Neutral detergent fiber	591	454	509	704	392
Ether extract	21.1	28.2	27.2	10.6	48.5
Starch	51.6	227	139	n.d.	369

DM, dry matter; n.d., not determined.

Chemical analysis

Feed ingredients of the experimental diets offered to the rumen inocula donors were collected in each experimental period for chemical analysis, including the HS and CS used as basal substrates in *in vitro* incubations. Thus, diets and basal substrates were analyzed for DM [method 934.01, 20] ash [method 942.05, 20], Kjeldahl N [method 954.01, 20], ether extract [method 920.39, 20] and NDF [with α -amylase and without sodium sulfite, and expressed exclusive of residual ash; 21, 22]. Crude protein (CP) was calculated as Kjeldahl N $\times$ 6.25. The starch content of CS and concentrate used in the experimental diets was determined on finely (0.5-mm) ground samples [23]. All chemical analyses were run in duplicate.

Rumen *in vitro* incubations

The ruminal contents of each animal were individually homogenized and strained through four layers of linen cloth. One part of each strained rumen fluid was diluted anaerobically with four parts of a buffer solution [24]. Treatments comprised two basal substrates (HS and CS) supplemented with two biochar sources (AFB and PPB) at two inclusion levels (5 and 10%, DM basis). Two hundred and fifty milligrams DM of treatments (237.5 or 225.0 mg substrate and 12.5 or 25.0 mg biochar, respectively, for 5 and 10% inclusion) or controls (basal substrate without substrate) were weighted, in 125 mL serum bottles (Sigma-Aldrich Inc., St. Louis, MO). Blank samples (without substrate or biochar) were also included. Fifty milliliters of buffered rumen fluid were added to each serum bottles (treatments, control and blanks). Anaerobiosis was achieved by flushing the bottles with O₂-free CO₂, and immediately sealing them with butyl rubber stoppers and aluminum caps. All treatments, controls, and blanks were incubated in triplicate (laboratory replicates), per incubation run and per experimental period, in a water bath at 39 °C. After 24 h of incubation, the fermentation was stopped by cooling the serum bottles into an ice water bath.

One incubation run of each individual inocula was carried out with 30 days interval.

Sampling, analysis, and calculations

Bottles were stepwise warmed up to 25 °C, and the fermentation gas volume was measured with a pressure transducer [25]. A gas sample was collected with a gas-tight syringe (SGE international PTY Ltd, Australia), and CH₄ analyzed by gas chromatography, using a GC-4000A (East & West Analytical Instruments, Inc, Beijing, China) equipped with a Shincarbon ST 100/120 micropacked column (Restek Corporation, Bellefonte, PA) and a thermal conductivity detector [26]. Total gas volume of samples was corrected for gas volume of blanks. CH₄ production was estimated from the CH₄ proportion in the gas sample corrected for blanks, the headspace volume of the incubation vessel and the ideal gas laws [27]. Gas and CH₄ yields were calculated as production volume (mL) per g of total (substrate and biochar) DM incubated.

After gas sampling, fermentation pH was immediately measured and fermentation media sampled for VFA and NH₃-N analysis. VFA were analyzed by gas chromatography using a Shimadzu GC-2010 Plus (Shimadzu Corporation, Kyoto, Japan) equipped with a capillary column (HP-FFAP, 30 m x 0.25 mm x 0.25 µm; Agilent Technologies, Santa Clara, CA), and a flame ionization detector. The individual VFA were identified and quantified as described by Maia *et al.* [28]. NH₃-N was analyzed by steam-distillation and the N content

determined by titration [20, ID 954.01]. Total VFA production and NH₃-N content were corrected for blanks.

Individual VFA and CH₄ (molar basis) were used in stoichiometric models to predict the reducing equivalents generated and the reducing equivalents consumed (expressed as $\mu\text{mol H}_2/\text{mL}$ fermentation media; Moss *et al.* [29]). Reducing equivalents generated were estimated as 2 acetic acid + 1 propionic acid + 4 butyric acid + 2 valeric acid + 2 *iso*-valeric acid, and reducing equivalents consumed as 2 propionic acid + 2 butyric acid + 1 valeric acid + 4 CH₄ [30]. Recovery (%) was calculated as reducing equivalents consumed/reducing equivalents generated x 100 [30]. Fermentation efficiency was calculated as (0.62 acetic acid + 1.09 propionic acid + 0.78 x butyric acid) / (acetic acid + propionic acid + butyric acid) x 100 and is based on the heats of combustion of glucose in the respective VFA [31].

Statistical analysis

Laboratory replicates were averaged before statistical analysis.

Data on the rumen fermentation parameters were analyzed using the MIXED procedure of SAS (2002; version 9.1, SAS Institute Inc., Carry, NC). The model included the fixed effects of biochar source (AFB and PPB) and level (5 and 10%), basal substrate (HS and CS), the interactions between biochar source and level, between biochar source and basal substrate and among biochar source, biochar level and basal substrate, the random effect of incubation run, and the random residual error. Differences among the means were identified using Tukey's multiple comparisons. Effects were considered significant when $p \leq 0.05$ and a trend when $0.05 < p \leq 0.10$. When the interaction was not significant, nor a tendency was observed ($p > 0.10$), it was removed from the model.

Student's *t*-test was used to compare the overall mean value of biochar supplementation with the overall mean value of control (basal substrate without biochar).

Table 6. Characterization of the agro-forestry biochar and the potato peel biochar used in the *in vitro* incubations.

	Agro-forestry biochar	Potato peel biochar
Carbonization conditions	600 °C, around 6 h	Heating rate of 10 °C/min until 500 °C, reaction time of 2 h, N ₂ flow of 150 mL/min. Yields around 30%
Surface area (S_{BET}), m²/g	94	<5
Total pore volume, cm³/g	0.05	n.q.
Micropore volume, cm³/g	0.03	n.q.
Mesopore volume, cm³/g	0.02	n.q.
pH_{PZC}	8.5	10.0
Ash content, % w/w	8.62	21.9
Elemental analysis, % w/w (as received basis)	C = 85.09; H = 1.81; N = 0.35; S = 0.00; O = 4.13	C = 65.74; H = 0.98; N = 1.86; S = 0.00; O = 9.52

n.q. not quantifiable

Results

In the present study, the interactions among biochar source, biochar level, and basal substrate and between biochar source and basal substrate were not significant nor a tendency was observed. Therefore, our study focused on the main effects of biochar source, biochar level and basal substrate and on the biochar source and biochar level interaction on gas production, CH₄ production, and rumen fermentation parameters, after 24-h *in vitro* incubation.

Chemical composition of substrates and biochars

The TMR offered to the cows' donors of rumen inocula had similar protein content and mainly differed on NDF and starch contents (Table 5). Regarding the basal substrates, NDF and CP contents were respectively 704 and 55.3 g/kg DM for HS and 392 and 77.4 g/kg DM for CS. Starch content of CS was 369 g/kg DM (Table 5).

AFB and PPB characterization are presented in Table 6. Apparent surface area (S_{BET}) was 94 and <5 m²/g for AFB and PPB, respectively. Ash content was lower for AFB than for PPB (8.62 and 21.9 w/w %, respectively). The pH_{PZC} value was 8.5 for AFB and 10.0 for PPB.

Basal substrates effects

Basal substrates had a significant effect on the rumen fermentation parameters from *in vitro* 24-h batch incubation (Table 7). CS decreased ($p < 0.001$) pH and acetic acid, *iso*-butyric, and valeric proportions, and increased ($p < 0.05$) total gas production and yield, CH₄ production and yield, total VFA production, butyric acid and caproic acid proportions, H₂ generated and consumed, recovery and fermentation efficiency. NH₃-N, propionic acid, *iso*-valeric acid and *iso*-caproic acid proportions and acetic:propionic ratio were unaffected ($p > 0.05$).

Table 7. Effect of control diet (basal substrate without biochar), biochar source and inclusion level, and substrate on the rumen fermentation parameters from *in vitro* 24-h batch incubation.

			Agro-forestry biochar		Potato peel biochar				<i>P</i>		Substrate			
	Control	<i>p</i>	5%	10%	5%	10%	SEM	S	L	SxL	Haylage	Corn silage	SEM	<i>P</i>
Total gas, mL	52.8	0.140	52.6	50.0	51.8	51.0	1.28	0.941	0.055	0.299	45.0	57.7	1.12	<0.001
Total gas, mL/g DM	221	0.240	222	211	218	214	5.4	0.999	0.555	0.311	190	242	4.71	<0.001
Methane, mL	4.02	0.001	4.67	4.71	4.71	4.43	0.465	0.541	0.535	0.411	3.75	5.51	0.445	<0.001
Methane, mL/g DM	16.9	0.001	19.7	19.8	19.8	18.6	1.96	0.522	0.534	0.403	15.9	23.1	1.88	<0.001
pH	6.36	0.666	6.36	6.37	6.36	6.36	0.014	0.980	0.349	0.324	6.39	6.33	0.013	<0.001
NH ₃ -N, mg/g DM	15.1	0.040	16.7	16.9	16.3	18.0	2.54	0.664	0.218	0.402	17.1	16.9	2.48	0.840
Total VFA, mmol/g DM	11.5	<0.001	11.0 ^b	10.2 ^a	11.2 ^b	10.9 ^b	0.82	0.001	<0.001	0.019	10.3	11.4	0.82	<0.001
Acetic acid, %	65.0	0.306	65.0 ^b	64.2 ^a	65.0 ^b	65.2 ^b	0.51	0.025	0.115	0.011	66.1	63.6	0.49	<0.001
Propionic acid, %	18.6	0.831	18.3	18.5	18.3	18.2	0.87	0.656	0.732	0.637	18.5	18.1	0.84	0.174
Iso-butyric acid, %	1.20	<0.001	1.22 ^a	1.29 ^b	1.23 ^a	1.23 ^a	0.010	<0.001	<0.001	<0.001	1.26	1.22	0.008	<0.001
Butyric acid, %	11.2	0.670	11.4	11.7	11.4	11.3	0.56	0.209	0.542	0.123	9.98	12.9	0.56	<0.001
Iso-valeric acid, %	2.21	0.002	2.26	2.39	2.27	2.27	0.075	0.243	0.195	0.194	2.30	2.30	0.068	0.881

Table 7 (cont.). Effect of control diet (basal substrate without biochar), biochar source and inclusion level, and substrate on the rumen fermentation parameters from *in vitro* 24-h batch incubation.

			Agro-forestry biochar		Potato peel biochar		<i>P</i>					Substrate		
	Control	<i>p</i>	5%	10%	5%	10%	SEM	S	L	SxL	Haylage	Corn Silage	SEM	<i>P</i>
Valeric acid, %	1.43	0.048	1.43 ^{ab}	1.48 ^b	1.43 ^{ab}	1.42 ^a	0.042	0.027	0.216	0.014	1.43	1.38	0.041	<0.001
Iso-caproic acid, %	0.0152	0.438	0.0146	0.0232	0.0145	0.0143	0.00428	0.299	0.329	0.306	0.015	0.019	0.0030	0.374
Caproic acid, %	0.384	0.980	0.378	0.389	0.379	0.369	0.0306	0.704	0.969	0.669	0.329	0.428	0.0251	<0.001
Enanthic acid, %	0.0209	0.937	0.0180	0.0195	0.0183	0.0216	0.00484	0.704	0.969	0.699	0.017	0.022	0.0046	0.052
Acetic:propionic acid ratio	3.56	0.866	3.61	3.52	3.61	3.62	0.194	0.048	0.592	0.479	3.59	3.59	0.186	0.998
H ₂ generated, mmol/L	110	<0.001	106 ^b	98.0 ^a	107 ^b	104 ^b	7.97	0.003	<0.001	0.048	96.2	111	7.92	<0.001
H ₂ consumed, mmol/L	33.7	0.001	32.5	30.6	32.9	31.9	1.85	0.019	<0.001	0.165	28.9	35.1	1.84	<0.001
Recovery, %	31.0	0.419	30.9	31.4	30.9	30.7	0.75	0.234	0.532	0.270	30.2	31.8	0.72	<0.001
Fermentation efficiency, %	73.1	0.978	73.0	73.2	73.0	72.9	0.36	0.391	0.593	0.319	72.9	73.2	0.34	0.049

DM, dry matter; NH₃-N, ammonia-N; VFA, volatile fatty acids; SEM, standard error of the mean; S, biochar source; L, biochar inclusion level; SxL, interaction between biochar source and biochar inclusion level.

^{a-d} Means in the same column followed by different letters differ ($p < 0.05$).

Biochar supplementation effects

When compared to control, biochar supplementation kept unaffected ($p > 0.05$) total gas production and yield, pH, acetic acid, propionic acid, butyric acid, *iso*-caproic acid, caproic acid, and enanthic acid proportions, acetic:propionic ratio, recovery, and fermentation efficiency (Table 7).

Biochar supplementation increased CH_4 production and yield ($p = 0.001$), $\text{NH}_3\text{-N}$ ($p = 0.040$), *iso*-butyric acid ($p < 0.001$), *iso*-valeric acid ($p = 0.002$), and valeric acid ($p = 0.048$) proportions. Conversely, biochar supplementation decreased total VFA production ($p < 0.001$), as well H_2 generated ($p < 0.001$) and H_2 consumed ($p = 0.001$, Table 7).

Biochar source and biochar inclusion level effects

The effects of biochar source and biochar inclusion level on the rumen fermentation parameters were sparse. Biochar source only affected acetic:propionic acid ratio ($p = 0.048$) and H_2 consumed ($p = 0.019$), with PPB promoting both parameters. Biochar level impacted in the H_2 consumed ($p < 0.001$) and total gas production ($p = 0.055$). All the other parameters were kept at similar levels for both biochar source and level ($p > 0.05$, Table 7).

Total VFA production ($p = 0.019$), acetic acid proportion ($p < 0.011$) and H_2 generated ($p = 0.048$) were lowest with AFB supplemented at 10%, no differences being observed among the other treatments. Conversely, AFB supplemented at 10% led to the highest *iso*-butyric acid proportion ($p < 0.001$, Table 7).

Discussion

Several strategies have been proposed to mitigate enteric CH_4 emissions from ruminants, the inclusion of feed supplements being one of the most common. In this research work, AFB and PPB were evaluated as dietary supplements for their effects on overall rumen fermentation parameters in 24-h *in vitro* batch incubations. The batch incubation technique was chosen since it is a simple method to evaluate the potential effect of novel feed ingredients and additives on the fermentation parameters prior to *in vivo* studies [32]. The reuse of industrial and forestry by-products into animal feed contribute to a more sustainable ruminant production sector pursuing a circular economy model.

Effect of basal substrate on rumen fermentation parameters

Basal substrates had a marked effect on the rumen fermentation parameters from *in vitro* 24-h batch incubation, with CS leading to a lower pH and to a higher total VFA, gas and CH_4 productions, H_2 generated and consumed, recovery and fermentation efficiency

than HS. Overall, these results reflect a greater extent of fermentation of CS relatively to HS. In addition, CS promoted a lower acetic acid proportion but failed to promote propionic acid proportion. Microbial fermentation of feeds in the rumen cause several end-products with numerous functions in the host animal [33]. The profile of these fermentation end-products is largely dependent on the feed nutrient composition. Roughage-based diets are known to promote the production of acetic acid whereas starch-rich diets decrease acetic acid proportion, increase propionic acid proportion and reduce fiber digestibility through decreased pH [34]. Both acetic acid and butyric acid promote CH₄ production, whereas propionic acid comprises a competitive pathway for H₂ use in the rumen, thus decreasing CH₄ production [29].

As acetic and butyric acids proportion were differently affected by substrates and that of propionic acid was unaffected, the promoted CH₄ production observed with CS when compared to HS may be explained by the higher extent of fermentation with CS, as supported by the increased VFA production and H₂ generated and consumed with this substrate. Results of CS on the rumen fermentation parameters herein reported strongly agree with those of Maia *et al.* [26].

Effect of biochar on methane and total gas production *in vitro*

In the present study, biochar supplementation increased CH₄ production and yield, and kept total gas production and yield unaffected when compared with control.

Effects of biochar on CH₄ production reported in literature are ambiguous, from neutral effect to an observed reduction of CH₄ being reported *in vitro* [8, 9, 16, 17, 35-38] and *in vivo* [39]. Such differences may reflect the different biomass and temperatures used to prepare biochars, which also confound comparison among studies. Leng *et al.* [9] suggested that the positive effects observed on CH₄ reduction are related to the high temperature (900 °C) used in biochar production. However, other studies reported a similar effect on CH₄ production of biochars prepared at 350 °C and 550 °C [17], and even higher methanogenesis reduction with biochar prepared at 550 °C than at 700 °C [40]. In our study, two biochar sources were used (AFB and PPB), prepared at two different temperatures (600 °C and 500 °C, respectively), yet the effects on CH₄ production and yield were similar. Additionally, the biochars used had different surface areas (AFB, 94 m²/g; PPB, <5 m²/g), that may reflect its capacity to trap gases, and pHPZC (pH at the point of zero charge), that measures the ionic state of functional groups which affect the adsorption capacity of biochar [41]. When compared to PPB, the higher S_{BET} of AFB (94 m²/g) may be related to the experimental pyrolysis conditions used to produce this biochar (higher temperature and residence time), allowing a higher devolatilization degree and porosity development.

Indeed, Calvelo Pereira *et al.* [17] found higher S_{BET} when biochars from pine chips and corn stover were obtained by slow pyrolysis at 550 °C than at 350 °C. Large surface to weight ratio (between 30 and 500 m²/g) was related to increased biofilm formation [35], thus suggesting that AFB could promote more microbial film formation in the rumen than PPB. Nevertheless, considering the similar performance of both biochars, other factors than S_{BET} may play a major role in total gas and CH₄ production and yield. Although AFB and PPB had different pH_{PZC} (8.5 and 10.0, respectively), both kept unchanged CH₄ production and yield. Similarly, no effects of biochar source on CH₄ were reported with alkaline biochars [pH > 8; 8, 17, 42] whereas acidic biochar (pH 4.8) lowered CH₄ production and yield [43].

Another possible explanation for the results obtained in the present study, comprises the use of rumen fluid, low in digesta particles, from cows not adapted to biochar. Indeed, the reduction of net CH₄ production was found with rumen fluid from cows adapted to biochar Leng *et al.* [36]. Multi-species biofilms have been described to establish on the external surface of the plant particles, also developing inside of the feed particles with the solubilization and utilization of the substrate [44]. These structures host the sequential degradation of complex structural and readily fermentable carbohydrates through several classes of microorganisms that complement each other. This sequential fermentation requires a low partial pressure of H₂ controlled by methanogenesis [36]. Inert materials, such as biochar, suspended in the fluid or associated with feed particles, will contribute to uptake the solubilized feed materials. This will also contribute to the diffusion of H₂ from the digestion of feed particles by providing a high surface area [36]. Biochar could thus create a suitable habitat for the association of microorganisms that will simultaneously allow a more efficient fermentation and promote CH₄ oxidation by joining archaeas and methanotrophic consortia [45]. Nevertheless, the biochar-microbe interaction is another key aspect that still needs further investigation to clarify its role not only in rumen fermentation but also in other similar anaerobic processes [46].

Effect of biochar on rumen fermentation *in vitro*

After 24-h incubation, biochar supplementation increased NH₃-N content when compared to control. An increase in NH₃-N could reflect higher proteolysis and deamination of substrate N or a decrease of NH₃-N incorporation into microbial protein [47]. The observed biochar effect could be due to both processes. Hence, effects of biochar on *iso*-butyric acid, *iso*-valeric acid and on valeric acid proportions suggest increased proteolysis and deamination, as these VFA are known to be originated from the microbial fermentation of valine, leucine, and proline, respectively [48]. Additionally, the similar gas production and lower VFA production observed with biochar supplementation than with control suggest a

reduced energy supply for microbial growth that can be related to lower microbial protein synthesis. Nevertheless, this rationale still needs to be confirmed by further research since protein metabolism is out of the scope of the present work.

Different effects of biochars sources on $\text{NH}_3\text{-N}$ have been reported in the literature. Some studies showed that the biochars' ability to adsorb $\text{NH}_3\text{-N}$ is inversely related to pyrolysis temperature at which biochar was produced [40, 49], as well as the effect of biomass source on cation adsorption [50]. Furthermore, ash content has been implicated in $\text{NH}_4^+\text{-N}$ sorption ability because when ash is washed off some of the exchangeable cations might be removed from the biochar surface [49, 50]. Despite the different pyrolysis temperatures to produce AFB and PPB biochar (600 and 500 °C, respectively) and their significantly different ash content (8.62 and 21.9% w/w, respectively), no effects were observed between biochar source (AFB and PPB) and between level (5 and 10%) on $\text{NH}_3\text{-N}$ content. Therefore, although the sorption capability of $\text{NH}_3\text{-N}$ was not measured, the AFB and PPB biochars seem to have similar $\text{NH}_3\text{-N}$ sorption ability.

Compared to control, biochar supplementation decreased total VFA production and increased *iso*-butyric acid, *iso*-valeric acid, and valeric acid proportions after 24-h *in vitro* fermentation. The decreased VFA production is consistent with the lower fermentable OM content of the biochar treatments, as AFB and PPB replaced either 5 or 10% of total DM incubated. However, previous studies have reported biochar to either increase VFA production or kept it unaffected [40, 42, 43]. Different effects on individual VFA profile related with biochar supplementation have also been described, including reduced propionic acid and butyric acid production [40], no effect on acetic [17] or on none of the individual VFA [42], and increased acetic acid, propionic acid and branched-chain fatty acids [43]. The contrasting results obtained in previous studies may be related to different biochar characteristics and inclusion levels that could influence the rumen microbial ecosystem and the profile of the fermentation end-products. Biochars from different biomasses have been supplemented from 0.5% [38, 43] up to 18.6% [17], DM basis, with a broad range of effects on *in vitro* rumen fermentation parameters. In our study, an average (5%) and a high (10%) biochar inclusion level were assessed, but the lower fermentable OM content, particularly at the highest inclusion level, may have contributed to the results herein presented. This highlights the importance of a full characterization of the biochar properties and methodological studies in order to better identify the factors by which biochar may affect the rumen fermentation parameters in both *in vitro* and *in vivo*.

No interaction was found between substrate and biochar source nor between substrate and biochar level on any of the parameters measured, agreeing with the results of Saleem *et al.* [43] and Teoh *et al.* [42]. Conversely, McFarlane *et al.* [15] suggested an improved fermentation when forages of intermediate quality but not of high quality, were

supplemented with biochar. In the present study, both corn silage and haylage presented lower CP content and lower and similar NDF content, respectively than the substrate used by McFarlane *et al.* [15; 9.8% CP and 70.4% NDF]. On the other hand, the substrates used by Saleem *et al.* [43] and Teoh *et al.* [42] presented higher CP and lower NDF contents compared to the study of McFarlane *et al.* [15]. This inconsistent behavior suggests that it is necessary to explore in more detail the effects of biochar on forages of different nutritive value.

The biochar source and inclusion level interaction observed on VFA production, acetic acid and *iso*-butyric acid proportions and H₂ generated was due to the AFB at 10%, no differences were observed among the other treatments, except for valeric acid. This is the first report of two biochars sources at two inclusion levels; in the previous works only different biochar sources or different biochar inclusion levels had been evaluated. However, biochar sources [15, 40] and biochar inclusion levels [43] were also reported to affect VFA production and individual VFA profile [15, 40]. Contrasting fermentation extent or profile may be due to differences in the biomasses used to produce biochar and pyrolysis conditions, resulting into biochars with different biochemical and structural properties. This will definitely influence characteristics as surface area, the pHPZC, and electroconductivity and thus affect the rumen microbial population, with impact on the multi-species biofilm formed and, ultimately, on the ruminal fermentation extent and profile.

Conclusions

This study assessed, for the first time, the effects of two biochar sources (AFB and PPB) supplemented at two levels (5 and 10%, DM basis) added to two basal substrates (HS and CS) on CH₄ production and major rumen fermentation parameters. Biochar supplementation, regardless of the source and level, increased CH₄ production and modified the fermentation pattern. Nevertheless, the biochar source and inclusion level lead to similar effects on rumen fermentation parameters after 24-h incubation, except for VFA production and H₂ produced and consumed. The similar behavior of the two different biochars suggests that other characteristics besides surface area and pyrolysis temperature may have important effects on the rumen fermentation pattern modulation, namely on CH₄ production and yield. Considering that the use of biochars as a feed supplement for decreasing CH₄ is still an emergent research topic, we believe that this work will add relevant information to the state of the art, by providing a systematic in-vitro comparison between two different biochars in controlled conditions. This work will also certainly impact on future studies that are still needed to clarify these complex interactions between biochar and the rumen ecosystem.

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Competing Interests

The authors declare there are no competing interests.

Author contributions

Ana R.F. Rodrigues performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft. Margarida R.G. Maia and Ana R.J. Cabrita conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft. Hugo M. Oliveira performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft. Maria Bernardo, Nuno Lapa, Isabel Fonseca, analyzed the data, prepared figures and/or tables, and approved the final draft. Henrique Trindade analyzed the data, authored or reviewed drafts of the paper, and approved the final draft. José L. Pereira analyzed the data, authored or reviewed drafts of the paper, and approved the final draft. António J.M. Fonseca conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.

Animal ethics

Animal procedures and methodologies were reviewed and approved by the Animal Ethics Committee of ICBAS-UP and licensed by the Portuguese Directorate-General of Food and Veterinary Medicine (permit #FT2014DGV 046412 ICB).

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Chapter Four

Ammonia and greenhouse gases emissions from cow's excreta are affected by feeding system, stage of lactation and sampling time

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Abstract

Decomposition of dairy cows' excreta on housing floor leads to ammonia and greenhouse gases production, yet factors affecting total emissions have not been fully disclosed. This work aimed to assess the impact of lactation stage, feeding system and sampling time on gaseous emission potential of cow's faeces and urine in laboratory chambers systems. Individual faeces and urine were collected from two groups of four cows, at peak and post peak lactation, from three commercial farms with distinct feeding systems: total mixed ration (TMR), total mixed ration plus concentrate at robot (TMR + robot), and total mixed ration plus concentrate in automatic feeders (TMR + AF). Samples were collected before a.m. (T8h), at middle day (T12h), and before p.m. (T17h) milking. In a laboratory chambers system, faeces and urine were mixed in a ratio of 1.7:1, and ammonia and greenhouse gases emissions were monitored during 48-h. Cumulative N–N₂O emissions were the highest in TMR + robot system, post peak cows and sampling time T17h. An interaction between stage of lactation and sampling time was detected for N–NH₃ and N–N₂O (g kg organic soluble N⁻¹) emissions. Post peak cows also produced the highest cumulative N–NH₃ emissions. Overall results contribute for the identification of specific on-farm strategies to reduce gaseous emissions from cows' excreta.

Keywords: Feed management; Greenhouse gases; NH₃; Sustainable milk production.

Introduction

Globally, dairy cattle houses are responsible for about 1.4 gigatons of CO₂-eq., which correspond to 20% of total livestock sector emissions [1]. Main emissions are CH₄, NH₃ and N₂O [2]. Methane is the second-largest contributor to climate radiative forcing, has a half-life of 12.4 years with a GWP of 28 in relation to CO₂ [3]. In the atmosphere, NH₃ plays a role in the formation of PM_{2.5}, can promote NH₄⁺ formation (an aerosol constituent) or can form N₂O a long-lived GHG, causing respiratory diseases, acid rain, eutrophication of soil and surface waters [4, 5].

Focusing on the dairy cattle building, the outset for CH₄, NH₃ and N₂O emissions are the gases released from the animals through respiration, eructation and flatulence and from excreted faeces and urine. Methane have a double origin, enteric rumen fermentation and manure decomposition. Enteric CH₄ production occurs by reduction of CO₂ with H₂ by the rumen methanogenic archaea, being mostly eructated [6], whereas CH₄ production from manure derives from its anaerobic decomposition as well as of the bedding material [7]. Ammonia is formed in the rumen from the hydrolysis of the degradable nitrogen (N) fraction of feed, thus being available to be used by microbes to synthesize microbial protein [8]. Excess of ruminal NH₃ is converted to urea in the liver and recycled to the rumen through saliva and epithelium or passed to the kidneys and excreted in urine [9, 10]. In the house floor, the urease produced by faecal microorganisms hydrolyses the urinary urea to form NH₄⁺/NH₃. Urease activity is determined by parameters such as pH, temperature, urinary urea content and house floor characteristics [11, 12]. Subsequently, depending on the air and floor temperature and wind speed, airflow drives the convective transfer of NH₃ from the excreta liquid phase to the air [12]. During the nitrification process NH₄⁺ can also be converted to NO₃⁻ which can be reduced to N₂ by the denitrification process, with nitric oxide and N₂O formed as intermediate products [13]. Besides, N₂O can also be formed in the rumen during fermentation of dietary proteins [14]. Thus, the chemical composition and physical characteristics of the feed, the N content and the energy-N balance of the diet and the physiological stage of the animal affect faeces and urine characteristics such as the amount of N excreted [15-17].

Methane, NH₃ and N₂O emission potential from dairy cattle excreta depends on several factors. Although urine application rate, temperature, floor design and excreta characteristics (such as protein concentration of the diet) on GHG and/or NH₃ emissions from faeces and urine have been studied [18-21], scarce information is available on the effect of feeding and milking frequency, and stage of lactation on gaseous emissions from faeces and urine. Moreover, the effect of excreta sampling time is not clear as studies varied from before and after morning milking [21], to total faeces collection up to 100 h [18, 19,

22]. More in-depth knowledge of the magnitude and impact of these factors on NH_3 and GHG emissions from dairy-cattle excreta would allow to adjust the management decisions towards a more sustainable milk production system. The present work addresses this issue by a threefold aim i) to assess the CH_4 , NH_3 , N_2O , and CO_2 emission potential of dairy cows' excreta from different feeding systems; ii) to detect differences in the CH_4 , NH_3 , N_2O , and CO_2 emission potential of excreta from animals in different stage of lactation; and iii) to study the CH_4 , NH_3 , N_2O , and CO_2 emissions from three distinct sampling times. The outputs of this work are anticipated to contribute for the improvement of dairy farms efficiency through its inclusion on future dairy farming methods recommendations.

Material and Methods

This study was conducted in three commercial dairy farms located in the northwest of Portugal. European guidelines for the care and use of animals were strictly followed (Directive, 2010/63/EU). Both urinary and faecal sampling were conducted by researchers in accordance with FELASA recommendations (category C) and following established veterinary guidelines.

Animal's housing and experimental design

Two groups of four healthy Holstein-Friesian dairy cows from each dairy farm were used in the study, one group in peak lactation (between 51 and 85 days in milk) and the other in post peak lactation (between 177 and 202 days in milk). All cows were fed a maize silage-based total mixed ration, but milking and feed systems differed among dairy farms. In farm 1, animals are fed a total mixed ration comprising 5.5 kg fresh grass, 30 kg maize silage, 1 kg wheat straw and 9 kg mixture of raw materials, cows being milked in parlour twice a day (TMR system). In farm 2, total mixed ration comprised 6 kg of fresh lucerne, 4 kg of grass silage, 30 kg of maize silage, 0.4 kg of wheat straw and 4 kg of mixture of raw materials, being animals milked in robot on average 3.6 times a day where they were supplemented a concentrate (4.6 kg for peak lactation and 5.0 kg for post peak lactation cows; TMR + robot system). In farm 3, the total mixed ration comprised 34 kg of maize silage, 1.7 kg of wheat straw and 6 kg of mixture of raw materials, with 4.3 kg and 3.1 kg of a commercial concentrate supplemented respectively to peak and post peak lactation cows, in automatic feeders, cows being milked in parlour twice a day (TMR + AF system). The chemical composition of total mixed rations and concentrates are presented in Table S1.

Urine and faeces sampling and processing

Faeces and urine samples were collected from each cow at three sampling times, a.m. milking (T8h), at middle day (T12h) and before p.m. milking (T17h). Urine samples were collected by vulva stimulation and faeces samples directly from cow's rectum. Individual samples collected at each sampling point were stored at 4 °C and transported to the laboratory. Composite samples of faeces and urine were made by stage of lactation (peak and post peak) and sampling time (T8h, T12h and T17h) in each feeding system. pH was immediately determined in urine samples using a pH meter. Faecal pH was determined in a faeces:ultrapure water (1:5 w/v) suspension according to 13037 [23]. Subsamples were taken after homogenisation and stored at -20°C until further analysis.

Laboratory chambers system experimental set-up

The laboratory chambers system (LCS, Figure 7) used to simulate a solid concrete cattle house floor was similar to the methodology described in detail by Misselbrook *et al.* [18] and Pereira *et al.* [22]. Briefly, LCS was composed by nine individual cylindric chambers (dimensions: 110 mm of diameter; 190 mm height; 0.42 mm of headspace) used simultaneously. Air was drawn through a tube system in each chamber by a vacuum pump. Polyethylene tubes (4 mm internal diameter) system were fitted to the cylindric chambers and sealed to exclude atmospheric N₂. The airflow rate of 4 L/min was regulated through a flow meter (Aalborg™ FT10201SAVN, Aalborg, Denmark). Acid traps containing 150 mL of 0.02M orthophosphoric acid were used to trap the NH₃ produced.

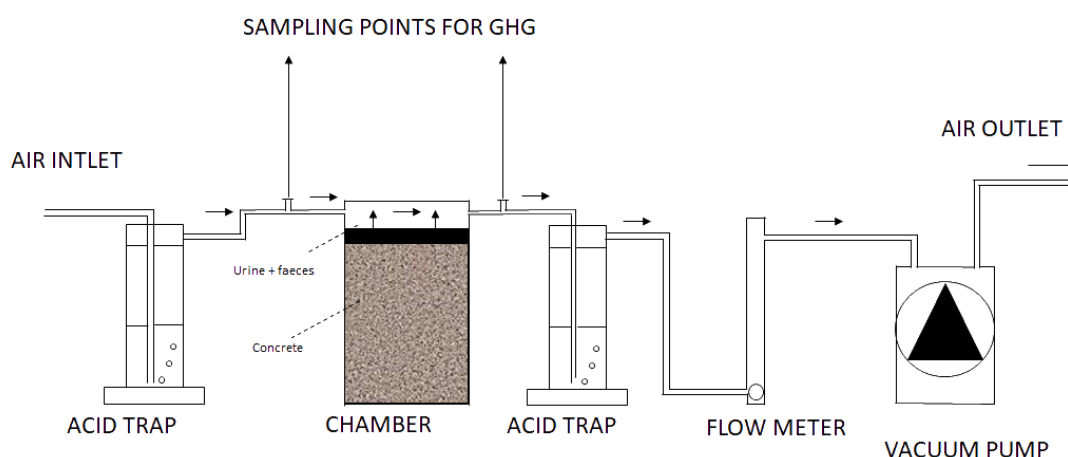


Figure 7 - Scheme of laboratory chambers system used to measure NH₃ and GHG emissions

Samples of each stage of lactation (peak and post peak), feeding system (TMR, TMR + robot and TMR + AF) and sampling time (T8h, T12h and T17h) were tested in 9

chambers in three runs of 48-h. The laboratory chambers system was housed in a climatic room (EVK 211, EVCO SPA, Belluno, Italy).

Faeces and urine samples were thawed and mixed in a ratio of 1.7:1 [24] corresponding to 13.6 g of faeces and 8 mL of urine, leaving an emitting layer of 1 mm thickness [18]. Ammonia, N₂O, CO₂ and CH₄ emissions were monitored during 48-h at 20 °C (± 1 °C). At the end of each experiment the main body of chambers was cleaned to avoid potential contamination between treatments.

Measurements of NH₃ and GHG concentrations and emissions calculation methodology

Ammonia content in air inlet was removed by the first acid trap positioned before each chamber. The second acid traps positioned after each chamber were used to determine NH₃ emissions and exchanged after 1, 3, 6, 12 h and every 12 h until 48 h. At the end of each sampling time, the volume of solution in the second acid trap was measured and a sample analysed for N-NH₄⁺ content (mg/L) by automated segmented-flow spectrophotometry [25].

Gas samples were collected at 0, 0.5, 1, 2, 4, 6, 8, 10, 12 h and every 12 h until 48 h on sampling points located immediately before and after the chamber. The first sampling point was used to collect a blank sample at each sampling time. In the second sampling point, the chamber headspace gas was collected with a 50 mL syringe (Omnifix®, Bbraun) and GHG quantified by gas chromatography using a GC-2010 Plus (Shimadzu, Japan) equipped with an electron capture ⁶³Ni detector (ECD), a thermal conductivity detector (TCD) and a flame ionization detector (FID) for N₂O, CO₂ and CH₄ analysis, respectively. The GC accuracy was 50 ppb–100 ppm for N₂O, 1 ppm to 1% for CO₂ and 0.1 ppm to 1% for CH₄. The NH₃ emissions were calculated according to Pereira *et al.* [22]. Carbon dioxide, CH₄ and N₂O fluxes in each sampling time were calculated using the gas concentration in the outlet air and discounting the concentration of each gas in the inlet air. The cumulative gas emissions between sampling times were calculated as the average of the flux between successive measurements multiplied by the time interval between sampling times. Cumulative GHG emissions were expressed as CO₂-equivalents using the conversion factors of 265 and 28 for N₂O and CH₄, respectively [26].

Chemical analysis

Faeces and urine samples were analysed for total C [27], soluble C [28], total N [29], ammoniacal-N [30], nitric-N [31], and dry matter (DM) content by oven drying (AOAC [32];

Ammonia and greenhouse gases emissions from cow's excreta are affected by feeding system, stage of lactation and sampling time

method 930.15) and freeze-drying, respectively. Urea-N content of the urine was also determined [33].

Statistical analysis

Statistical analyses were performed on the data using Statistix 10.0 software program 2015 (Tallahassee, FL, USA). The split-plot analysis of variance (ANOVA) was used to analyse the effect of feeding system, stage of lactation and sampling time and the interactions between feeding system and stage of lactation, feeding system and sampling time, stage of lactation and sampling time, and among feeding system, stage of lactation and sampling time on cumulative NH_3 and GHG fluxes and faecal and urinary characteristics. Significant differences of the means among treatments and their interactions were determined by Tukey's multiple comparisons. Effects were considered significant when $p \leq 0.05$ and a trend when $0.05 < p \leq 0.10$. When the interaction was not significant, nor a tendency was observed ($p > 0.10$), it was removed from the model.

Results

Cows' intake, milk yield and feed efficiency

Dry matter intake, milk yield and feed conversion efficiency of cows in peak and post peak lactation from the three feeding systems are presented in Table S2. Dry matter intake varied from 20.3 kg/day in TMR system to 21.7 kg/day in post peak lactation cows of TMR + robot system. Average milk yield in peak production cows was 38.1 kg/day and of post peak 37.3 kg/day, the highest milk yield being observed for post peak cows from TMR + robot system (48.1 kg/day). Average energy-corrected milk (kg/kg dry matter intake) in peak production cows was 1.68 and of post peak 1.80, with post peak cows from TMR + robot system presenting the highest value.

Faeces and urine characteristics

The characteristics of faeces and urines collected are presented as supplementary material (Table S3 to Table S6). The feeding system mostly affected the urinary characteristics with TMR + robot presenting lower DM ($p = 0.044$), total C ($p = 0.022$), total N ($p = 0.005$), ammoniacal-N ($p = 0.011$), ureic-N ($p = 0.014$) and organic soluble N ($p = 0.005$) than TMR and TMR + AF (Table S3). Stage of lactation had no effect on urinary characteristics and only tended to affect faecal pH ($p = 0.075$, Table S4). Sampling time affected faecal ammoniacal-N, being the highest at T17h and the lowest at T12h ($p = 0.043$), and tended to affect faecal pH and total C, and urinary organic soluble N and total N (Table

S5). The interaction between stage of lactation and sampling time only tended to affect urinary ammoniacal-N ($p = 0.099$, Table S6).

Effect of feeding system on potential GHG and NH_3 emissions

The feeding system affected ($p < 0.05$) cumulative N– N_2O emissions (expressed as g kg ammoniacal-N⁻¹, ureic N, and organic soluble N, Table 9), TMR + robot presenting the highest emissions, with no differences observed between TMR and TMR + AF. However, no differences were detected among feeding systems for cumulative N– N_2O emissions (g/kg total N, Table 8). Total mixed ration plus concentrate in automatic feeders presented the highest cumulative N– NH_3 emission (g/kg excreta), followed by TMR and the lowest emission being observed for TMR + robot ($p < 0.05$, Table 8). Total mixed ration and TMR + AF presented the highest cumulative N– NH_3 emission (g kg/total N) and TMR + robot the lowest ($p < 0.05$, Table 8). No differences were detected among feeding systems for cumulative C– CH_4 emissions (g/kg excreta, total C, and organic C, Table 8), N_2O global warming potential (g CO_2 -eq/kg excreta) and contribution to total of GHG emission (%; Table 8).

Effect of stage of lactation on GHG and NH_3 emissions

Stage of lactation had a significant effect ($p < 0.05$) on cumulative N– N_2O emissions (g kg total N⁻¹ and ammoniacal-N) and on cumulative N– NH_3 (g/kg excreta and total N) with post peak lactation animals presenting the highest emissions (Table 9). Global warming potential from N_2O tended to be higher in post peak lactation cows ($p = 0.085$). Fluxes of N– N_2O (mg/h/kg total N and ammoniacal-N) from post peak lactation were higher than peak lactation, with peak lactation cows presenting higher flux at 2-h of deposition ($p < 0.05$, Figure 8 a and b). Similarly, fluxes of NH_3 (mg N/h/kg total N and N– NH_4) were higher from post peak than peak lactation excreta, higher fluxes being observed up to 6-h after excreta deposition of post peak cows ($p < 0.05$, Figure 8 c and d).

Ammonia and greenhouse gases emissions from cow's excreta are affected by feeding system, stage of lactation and sampling time

Table 8. Effect of feeding system on cumulative N-N₂O and N-NH₃ emissions, global warming potential of CH₄ and N₂O, and percentage of CH₄ and N₂O contribution to total GHG emission.

	Feeding system				
	TMR	TMR+robot	TMR+AF	SEM	<i>p</i>
<i>Cumulative N-N₂O</i>					
g/kg total N	1.43	1.50	1.36	0.397	0.407
g/kg ammoniacal-N	68.6 ^b	104 ^a	68.1 ^b	20.83	<0.001
g/kg ureic N	19.6 ^b	29.1 ^a	16.4 ^b	5.72	<0.001
g/kg organic soluble N	8.64 ^b	11.4 ^a	8.00 ^b	2.470	<0.001
<i>Cumulative N-NH₃</i>					
g/kg excreta	2.44 ^b	1.62 ^c	2.74 ^a	0.137	<0.001
g/kg total N	146 ^a	97.5 ^b	152 ^a	7.83	<0.001
g/kg soluble organic N	875 ^a	746 ^b	893 ^a	47.0	0.002
<i>Cumulative C-CH₄</i>					
g/kg excreta	0.031	0.033	0.039	0.0075	0.549
g/kg total C	0.119	0.127	0.154	0.0294	0.408
g/kg organic C	42.0	43.1	44.6	9.99	0.958
<i>Global warming potential</i>					
CH ₄ (g CO ₂ .eq/kg excreta)	1.17	1.23	1.44	0.281	0.549
N ₂ O (g CO ₂ .eq/kg excreta)	20.0	20.8	20.1	5.25	0.838
<i>Contribution to total GHG</i>					
CH ₄ (%)	6.58	7.06	7.15	2.324	0.890
N ₂ O (%)	93.4	92.9	92.8	2.32	0.890

TMR – feeding system with only total mixed ration; TMR+robot - feeding system with total mixed ration plus concentrate at robot; TMR+AF - feeding system with total mixed ration plus concentrate at automatic feeders; SEM - standard error of the mean; Values followed by different lowercase superscript letters in rows indicate significantly different means ($p < 0.05$), according to the Tukey' test.

Ammonia and greenhouse gases emissions from cow's excreta are affected by feeding system, stage of lactation and sampling time

Table 9. Effect of stage of lactation on cumulative N-N₂O and N-NH₃ emissions, global warming potential of CH₄ and N₂O, and percentage of CH₄ and N₂O contribution to total GHG emission.

Stage of lactation				
	peak	post peak	SEM	<i>p</i>
Cumulative N-N₂O				
g/kg total N	1.32	1.54	0.367	0.015
g/kg ammoniacal-N	74.1	86.7	20.65	0.030
g/kg ureic N	21.6	21.8	5.67	0.869
g/kg organic soluble N	9.03	9.66	2.447	0.355
Cumulative N-NH₃				
g/kg excreta	2.07	2.47	0.128	<0.001
g/kg total N	117	146	7.3	<0.001
Cumulative C-CH₄				
g/kg excreta	0.0462	0.0223	0.00698	<0.001
g/kg total C	0.179	0.0867	0.02722	<0.001
g/kg organic C ⁻¹	60.4	26.0	9.31	<0.001
Global warming potential				
CH ₄ (g CO ₂ .eq/kg excreta)	1.73	0.833	0.2606	0.001
N ₂ O (g CO ₂ .eq/kg excreta)	19.3	21.4	5.21	0.085
Contribution to total GHG				
CH ₄ (%)	9.29	4.57	0.002	<0.001
N ₂ O (%)	90.7	95.4	0.002	<0.001

SEM - standard error of the mean;

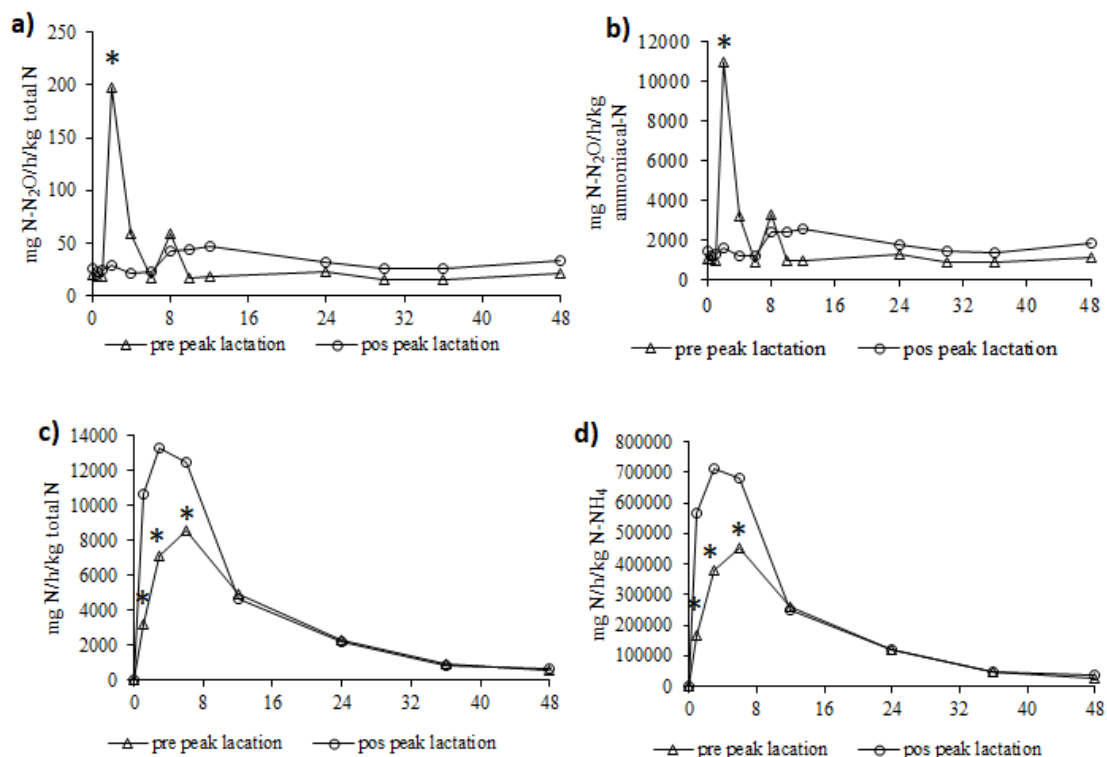


Figure 8 -Stage of lactation effect on N₂O emissions fluxes expressed as a) mg N-N₂O/h/kg total N and b) mg N-N₂O/h/kg ammoniacal-N and on NH₃ emission fluxes expressed as c) mg N/h/kg/ total N and d) mg N/h/kg N-NH₄. *Represents time points with statistically different (*p* < 0.05) peak and post peak fluxes

Effect of sampling time on GHG and NH₃ emissions

Sampling time had a significant effect ($p < 0.05$) on cumulative N–NH₃ (g excreta and total N) emissions (Table 10). Cumulative N–N₂O emissions (g kg ammoniacal-N⁻¹) were promoted at T17h ($p < 0.05$; Table 11), but fluxes of N–N₂O (mg/h/kg ammoniacal-N) were similar among sampling times ($p > 0.05$; Figure 9).

Table 10. Effect of sampling time on cumulative N–N₂O and -NH₃ emissions, global warming potential of CH₄ and N₂O, and percentage of CH₄ and N₂O contribution to total GHG emission.

	Sampling time				
	T8h	T12h	T17h	SEM	<i>p</i>
<i>Cumulative N–N₂O</i>					
g/kg total N	1.41	1.35	1.53	0.370	0.243
g/kg ammoniacal-N	76.8 ^b	67.8 ^b	96.6 ^a	20.83	<0.001
g/kg ureic N	20.9	19.8	24.4	5.72	0.055
g/kg organic soluble N	9.87	8.75	9.41	2.470	0.403
<i>Cumulative N–NH₃</i>					
g/kg excreta	2.28 ^b	2.59 ^a	1.93 ^c	0.137	<0.001
g/kg total N	131 ^{ab}	146 ^a	119 ^b	7.8	<0.001
g/kg organic soluble N	864 ^a	928 ^a	722 ^b	47.0	<0.001
<i>Cumulative C–CH₄</i>					
g/kg excreta	0.0425	0.0326	0.0277	0.00752	0.103
g/kg total C	0.166	0.125	0.107	0.0029	0.098
g/kg organic C	52.4	37.6	39.6	9.99	0.211
<i>Global warming potential</i>					
CH₄ (g CO₂-eq/kg excreta)	1.59	1.22	1.03	0.281	0.103
N₂O (g CO₂-eq/kg excreta)	20.3	20.0	20.7	5.25	0.902
<i>Contribution to total GHG</i>					
CH₄ (%)	8.34	6.86	5.60	2.32	0.113
N₂O (%)	91.7	93.1	94.4	2.32	0.113

T8h – sampling time before a.m. milking; T12h – sampling time at middle day; T17h - sampling time before p.m. milking; SEM - standard error of the mean; Values followed by different lowercase superscript letters in rows indicate significantly different means ($p < 0.05$), according to the Tukey' test.

Ammonia and greenhouse gases emissions from cow's excreta are affected by feeding system, stage of lactation and sampling time

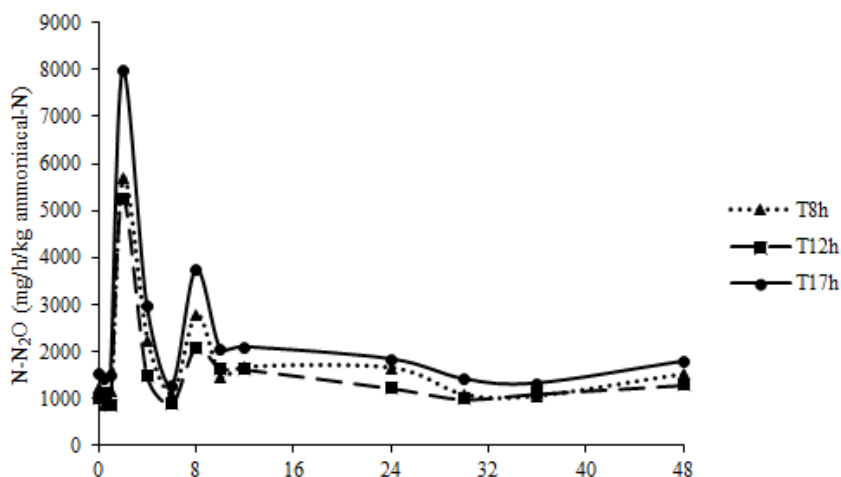


Figure 9 - Sampling time effect on excreta N-N₂O emission fluxes (mg/h/kg ammoniacal-N)

Cumulative C-CH₄ emissions (g/kg total C) tended to be higher at T8h (Table 10). No differences were detected among sampling times for cumulative N-N₂O (g/kg organic soluble N) and C-CH₄ emissions (g/kg excreta, and organic C), GWP CH₄ and N₂O (g CO₂-eq kg⁻¹ excreta), and N₂O and CH₄ contribution relatively to the total of GHG emission (%; Table 101).

Interaction between stage of lactation and sampling time on cumulative GHG and NH₃ emissions

The effects of stage of lactation and sampling time interaction on GHG and NH₃ emissions are presented in Table 11. Cumulative N-NH₃ emissions (g/kg soluble organic N), were the highest in post peak cows at T8h and T12h and the lowest in the remaining treatments ($p = 0.001$). Cumulative N-N₂O emissions (g/kg organic soluble N) were the highest from peak lactation cows at T17h and lowest from peak lactation cows at T12h emissions, not differing from the other treatments ($p = 0.004$). Global warming potential from N₂O (g CO₂-eq/kg excreta) was not affected ($p = 0.452$). Cumulative C-CH₄ emissions (g/kg excreta, total and organic C) were the highest in peak lactation cows at T8h, not differing from peak lactation cows at T17h, and the lowest in the remaining treatments ($p < 0.05$). A similar pattern was followed by GWP from CH₄ (g CO₂-eq/kg excreta; $p = 0.007$). Excreta from peak lactation cows at T8h had the highest CH₄ (%) contribution relatively to the total of GHG emissions, while post peak lactation cows at T17h had the lowest ($p = 0.033$). Conversely, peak lactation cows at T8h interaction had the lowest N₂O (%) contribution relatively to the total of GHG emissions while the highest was from post peak lactation cows at T17h ($p = 0.033$).

Table 11. Interaction between stage of lactation and sampling time on cumulative C-CH₄, and N-N₂O emissions, on global warming potential of CH₄ and N₂O, and percentage of CH₄ and N₂O contribution to total GHG emission.

	peak lactation			post peak lactation				
	T8h	T12h	T17h	T8h	T12h	T17h	SEM	<i>p</i>
<i>Cumulative N-N₂O</i>								
g/kg total N	1.39	1.16	1.40	1.43	1.54	1.66	0.377	0.312
g/kg ammoniacal-N	68.4	56.6	97.1	85.1	78.9	96.0	21.4	0.215
g/kg ureic N	21.8	17.1	25.7	20.0	22.4	23.1	5.88	0.093
g/kg organic soluble N	9.04 ^{ab}	7.27 ^b	10.8 ^a	10.7 ^{ab}	10.2 ^{ab}	8.03 ^{ab}	2.537	0.004
<i>Cumulative N-NH₃</i>								
g/kg excreta	2.25	1.36	2.59	2.63	1.88	2.89	0.160	0.392
g/kg total N	121	133	98.0	140	159	139	9.1	0.234
g/kg soluble organic N	712 ^b	804 ^b	728 ^b	1016 ^a	1052 ^a	715 ^b	55.0	0.001
<i>Cumulative C-CH₄</i>								
g/kg excreta	0.0651 ^a	0.0321 ^b	0.0414 ^{ab}	0.0199 ^b	0.0331 ^b	0.0140 ^b	0.00895	0.007
g/kg total C	0.254 ^a	0.123 ^b	0.161 ^{ab}	0.0789 ^b	0.127 ^b	0.0539 ^b	0.03507	0.008
g/kg organic C	80.3 ^a	40.0 ^{bc}	61.0 ^{ab}	24.6 ^{bc}	35.4 ^{bc}	18.2 ^c	11.80	0.019
<i>Global warming potential</i>								
CH ₄ (g CO ₂ .eq/kg excreta)	2.43 ^a	1.20 ^b	1.55 ^{ab}	0.743 ^b	1.24 ^b	0.521 ^b	0.3340	0.007
N ₂ O (g CO ₂ .eq/kg excreta)	20.2	18.0	19.6	20.4	22.0	22.0	5.35	0.452
<i>Contribution to total GHG</i>								
CH ₄ (%)	12.1 ^a	7.27 ^{abc}	8.47 ^{ab}	4.54 ^{bc}	6.46 ^{bc}	2.72 ^c	2.493	0.033
N ₂ O (%)	87.9 ^c	92.7 ^{abc}	91.5 ^{bc}	95.5 ^{ab}	93.5 ^{ab}	97.3 ^a	2.49	0.033

T8h – sampling time before a.m. milking; T12h – sampling time at middle day; T17h - sampling time before p.m. milking; Values followed by different lowercase superscript letters in rows indicate significantly different means (*p* < 0.05), according to the Tukey' test.

Discussion

Effect of feeding system on cumulative GHG and NH₃ emissions

The feeding system had a marked effect on N₂O and NH₃ emissions. Excreta from TMR + robot led to higher N–N₂O emissions (g kg ammoniacal-N⁻¹, ureic N, and organic soluble N) than TMR and TMR + AF. However, the urinary content of TMR + robot presented the lowest ureic-N and ammoniacal-N excretions of the three dairy systems suggesting a better synchronism on the availability of N and energy in the rumen. Dietary composition has a significant impact on N excretion [34], as synchronization of N and energy availability for rumen microbial protein synthesis prevents concentration of NH₃ above the optimum that leads to a high urea excretion [35, 36]. Although nutrient intake was not determined in the present study, results suggest that the lowest urea production and/or excretion in TMR + robot may be due to a better synchronism of N and energy in the diet. In this context, the higher N₂O emissions from TMR + robot compared to TMR and TMR+ AF may be a sum of different emission pathways. Firstly, and the most likely hypothesis, TMR + robot excreta may have had a more favourable environment for denitrification with N₂O being formed as an intermediate product. In the anaerobic respiration process microorganisms may have used NO₃⁻ as final electron acceptor in the presence of labile C more efficiently. Secondly, the aerobic conditions of the surface emitting layer may have promoted the N₂O production from N–NH₃ volatilization and from the hydrolysis of urea to NH₃. Moreover, N₂O emissions could also have derived from the release of N₂O, produced as a product of the digestive process at the lower gut, that might have been trapped in the excreta. Indeed, [37] suggested that the solved N₂O trapped in lucerne silage was transferred into the rumen, being responsible for the higher production of N₂O, when compared to those of cows fed grass hay or corn silage. No lucerne silage was used in the TMR + robot diet but we may not discard a putative effect of the fresh lucerne used exclusively in this feeding system. However, studies on the mechanism behind N₂O formation and excretion in the rumen or colon are scarce [38] and further research on the topic is needed to confirm this hypothesis. The greatest ammoniacal-N content of TMR and TMR + AF urines may explain the higher NH₃ emissions from these dairy feeding systems compared to TMR + robot, as this N form is susceptible to NH₃ volatilization losses [39]. In addition, the greatest organic soluble N content of TMR and TMR + AF urines and the aerobic conditions of the emitting layer may have led to mineralization of organic N into NH₃ [40].

Effect of stage of lactation on cumulative GHG and NH₃ emissions

Dairy herd comprises animals at all stages of the lactation cycle and with different individual productivity. Cows' lactation curve describes changes in milk production and milk constituents on the course of lactation and can be a representation of the herd management and breeding practices [41]. Genetic factors, physiological and metabolic conditions, digestive functions, and diet affect the proportion of feed intake converted by the animal to milk yield [42]. In the present study, cows in peak lactation and post peak lactation presented similar dry matter intake, milk yield and consequently similar feed efficiency. However, excreta from post peak lactation animals produced higher cumulative emissions of N–N₂O (g/kg total N and ammoniacal-N) and N–NH₃ (g/kg excreta and total N) than excreta from peak lactation animals although faecal pH only tended to be higher for peak lactation cows. Fluxes profiles of the 48-h experiment may add extra information to explain these differences.

Indeed, excreta from both groups of animals presented different N–N₂O flux patterns (mg/h/kg total N and ammoniacal-N) in the first hours of deposition. Excreta from peak lactation cows presented a first peak of emission at 2-h after excreta deposition, which may suggest a release of N₂O trapped in peak lactation faeces either as result of cow's metabolism or due to faecal characteristics like porosity or texture that may have allowed the gas release. A second (non-significant) emission peak occurred at the 8-h of deposition, being relatively stable until the end of the 48-h. A similar N₂O emission pattern was observed in simulated floors [22], soil [43] and dairy manure [44]. Excreta from post peak lactation group presented a lower N–N₂O emission at 2-h deposition but cumulative gases were higher than that of peak lactation cows.

Effect of sampling time on cumulative GHG and NH₃ emissions

Diurnal variations of N₂O emissions from barns and barn floor are described in several studies with a reported increase in the late afternoon [45, 46], being barn temperature correlated with N₂O emission peak [46]. In our study, cumulative N–N₂O emissions (g/kg ammoniacal-N) were highest at T17h whereas faecal ammoniacal-N content tended to be lower, and no differences were detected for urinary ammoniacal-N content. Moreover, N–N₂O (mg/h/kg ammoniacal-N) fluxes demonstrated a peak after 2-h deposition of N–N₂O numerically higher for T17h than for T8h and T12h, which may suggest more trapped N₂O in T17h excreta. However, temperature of indoor housing was not recorded at sampling time. Another possible explanation may rely on LCS aerobic

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environment, with T17h excreta presenting a more favourable environment for ammonia-oxidizing bacteria and ammonia-oxidizing archaea and higher N_2O emission during nitrification of NH_4^+ [47, 48].

Moreover, the higher cumulative $\text{N}-\text{N}_2\text{O}$ emission from T17h may explain the lowest NH_3 emission (g/kg excreta, total N, and organic soluble N) compared to the other sampling times. Also, T12h presented the highest faecal ammoniacal-N and tended to have higher urinary organic soluble N, followed by T8h with T17h having the lowest contents. A possible higher urease activity of T12h excreta may explain the volatilization of NH_3 at this sampling time. Besides, T12h excreta characteristics, such as porosity or texture could have affected the dissolution of gaseous NH_3 or promoted the mineralization of organic N into NH_3 [40].

Effect of the interaction between stage of lactation and sampling time on cumulative GHG and NH_3 emissions

The $\text{N}-\text{NH}_3$ and $\text{N}-\text{N}_2\text{O}$ emissions were only affected by the interaction between stage of lactation and sampling time when expressed as g/kg organic soluble N. No differences were observed in excreta characteristics except for a tendency for a lower urinary ammoniacal-N. The ammoniacal-N present in excreta may have promoted N_2O emission by nitrification and/or denitrification [49]. The denitrification process is expected to be the highest at pH range of 7.0–7.5 [50], the anaerobic environment also promoting CH_4 production [3]. Excreta from peak lactation cows at T17h followed this pattern, presenting the highest $\text{N}-\text{N}_2\text{O}$ emission, lower $\text{N}-\text{NH}_3$ and higher $\text{C}-\text{CH}_4$ emissions (g/kg excreta, total C, organic C). However, different patterns were observed in the other treatments, further studies being needed to elucidate the interaction between stage of lactation and sampling time.

Environmental conditions are known to affect cow's voluntary intake, rumination time, milk production and the diurnal feeding pattern [51-53]. In this study, faeces and urine sampling were performed during the summer of 2017, classified as a hot and extremely dry season [54]. Individual dry matter intake was not assessed but results suggest that peak lactation animals may have ingested a higher amount of feed and/or had a higher ruminal rate of passage, leading to more fermentable OM being present in the excreta [52, 55, 56]. This hypothesis could promote anaerobic fermentation of degradable-C compounds of faeces by methanogenic archaea presented in faeces explaining the higher cumulative $\text{C}-\text{CH}_4$ emissions (g/kg excreta, total C, and organic C). The higher CH_4 emissions may explain the highest GWP for CH_4 from peak lactation at T8h followed by peak lactation at T17h, with a cumulative emission of 2.43 and 1.55 (respectively, g $\text{CO}_2\text{-eq/kg}$ excreta); no differences being found for GWP for N_2O .

Conclusions

The present study assessed the effect of feeding system, stage of lactation and sampling time and their interactions on the emission potential of GHG and NH_3 from dairy cows' excreta in LCS. Feeding systems were shown to affect cumulative $\text{N-N}_2\text{O}$ emissions, TMR + robot presenting the highest emission potential. Conversely, TMR and TMR + AF presented the highest N-NH_3 emissions when compared to TMR + robot. Stage of lactation differently affected N_2O and NH_3 emissions, with excreta from post peak animals leading to higher cumulative emissions of $\text{N-N}_2\text{O}$ and N-NH_3 , and different fluxes trends, suggesting that faecal textural or porosity characteristics may have a marked effect on N_2O release. Moreover, an interaction between stage of lactation and sampling time was observed for N-loss as N-NH_3 and $\text{N-N}_2\text{O}$ from the excreta organic soluble N form. The outcome of this study adds relevant information to the state of the art on the topic. Practical guidelines on NH_3 and GHG emissions adjusted to a farm level basis could be a key strategy to reduce carbon footprint and increase competitiveness and resilience of the dairy sector if adopted to policy measures and regulations.

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Author contributions

Ana Rodrigues: Investigation; Formal analysis; Writing - original draft. Margarida Maia: Conceptualization; Investigation; Writing - review & editing. Carla Miranda: Investigation; Writing - review & editing. Ana Cabrita: Conceptualization, Writing - review & editing. António Fonseca: Conceptualization, Resources, Writing - review & editing. José

Pereira: Conceptualization; Writing - review & Editing. Henrique Trindade: Conceptualization; Supervision; Resources, Formal analysis; Writing - review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary material

Table S1. Chemical composition (g/kg dry matter) of cow's diets used in the studied feeding systems.

	TMR	TMR+robot	TMR+AF
<i>Total mixed ration</i>			
Dry matter (g/kg)	487	387	410
Organic matter	927	928	943
Crude protein	163	145	145
Neutral detergent fibre	373	409	363
Starch	260	180	296
<i>Concentrate</i>			
Dry matter (g/kg)	-	899	905
Organic matter	-	888	885
Crude protein	-	307	275
Neutral detergent fibre	-	195	222
Starch	-	179	178

TMR- feeding system with only total mixed ration; TMR+robot - feeding system with total mixed ration plus concentrate at robot; TMR+AF - feeding system with total mixed ration plus concentrate at automatic feeders.

Ammonia and greenhouse gases emissions from cow's excreta are affected by feeding system, stage of lactation and sampling time

Table S2. Dry matter intake (kg/day), milk yield (kg/day), energy corrected milk (kg/kg dry matter intake) and feed efficiency (kg/kg dry matter intake) of cows according to feeding system and stage of lactation.

	TMR		TMR+robot		TMR+AF		Average	
	peak	post peak	peak	post peak	peak	post peak	peak	post peak
Dry matter intake	20.3		21.3	21.7	21.6	20.5	21.1	20.8
Milk yield	34.5	32.0	42.4	48.1	37.4	31.8	38.1	37.3
Energy corrected milk	33.8	32.4	35.2	46.2	37.3	34.2	35.5	35.9
Feed conversion efficiency								
Milk	1.70	1.57	1.99	2.22	1.73	1.55	1.81	1.79
Energy corrected milk	1.67	1.59	1.65	2.13	1.72	1.67	1.68	1.80

TMR - feeding system with only total mixed ration; TMR+robot - feeding system with total mixed ration plus concentrate at robot; TMR+AF - feeding system with total mixed ration plus concentrate at automatic feeders. Energy-corrected milk calculated as: $(0.324 \times \text{kg of milk}) + (12.95 \times \text{kg of milk fat}) + (7.20 \times \text{kg of milk protein})$.

Ammonia and greenhouse gases emissions from cow's excreta are affected by feeding system, stage of lactation and sampling time

Table S3. Effect of feeding system on faecal and urinary characteristics

	Feeding System			SEM	<i>p</i>
	TMR	TMR+robot	TMR+AF		
<i>Faeces</i>					
pH	6.89	6.95	6.94	0.05	0.721
Dry matter (%)	13.4	14.0	13.7	0.17	0.183
Total C (%)	40.9 ^a	41.0 ^a	39.0 ^b	0.18	0.003
Organic soluble C (%)	0.13	0.12	0.15	0.01	0.154
Total N (%)	2.23	2.39	2.35	0.05	0.180
Ammoniacal-N (mg/kg)	223	190	204	14.3	0.366
Nitric-N (mg/kg)	282	318	483	54.3	0.115
Organic soluble N (%)	0.04	0.12	0.02	0.05	0.365
<i>Urine</i>					
pH	8.56	8.53	8.50	0.02	0.204
Dry matter (%)	5.80 ^{ab}	4.65 ^b	6.22 ^a	0.30	0.044
Total C (%)	1.48 ^a	0.98 ^b	1.38 ^a	0.08	0.022
Total N (%)	0.75 ^a	0.49 ^b	0.86 ^a	0.04	0.005
Ammoniacal-N (mg/L)	594 ^a	355 ^b	649 ^a	38.0	0.011
Ureic-N (%)	0.34 ^a	0.24 ^b	0.40 ^a	0.02	0.014
Organic soluble N (%)	0.69 ^a	0.46 ^b	0.79 ^a	0.03	0.005

TMR- feeding system with only total mixed ration; TMR+robot – feeding system with total mixed ration plus concentrate at robot; TMR+AF – feeding system with total mixed ration plus concentrate at automatic feeders; SEM – Standard error of the mean. Values followed by different lowercase superscript letters in rows indicate significantly different means ($p < 0.05$).

Ammonia and greenhouse gases emissions from cow's excreta are affected by feeding system, stage of lactation and sampling time

Table S4. Effect of the stage of lactation on faecal and urinary characteristics.

	Stage of lactation		SEM	<i>p</i>
	Peak	Post peak		
<i>Faeces</i>				
pH	7.00	6.86	0.042	0.075
Dry matter (%)	13.5	13.8	0.140	0.263
Total C (%)	40.3	40.3	0.149	0.961
Organic soluble C (%)	0.124	0.139	0.006	0.141
Total N (%)	2.37	2.27	0.042	0.168
Ammoniacal-N (mg/kg)	209	202	11.67	0.675
Nitric-N (mg/kg)	341	380	44.34	0.565
Organic soluble N (%)	0.039	0.082	0.037	0.450
<i>Urine</i>				
pH	8.52	8.53	0.016	0.710
Dry matter (%)	5.65	5.47	0.241	0.630
Total C (%)	1.36	1.21	0.064	0.183
Total N (%)	0.724	0.671	0.030	0.276
Ammoniacal-N (mg/L)	549	517	31.05	0.501
Ureic-N (%)	0.313	0.339	0.017	0.351
Organic soluble N (%)	0.671	0.621	0.028	0.276

SEM – Standard error of the mean.

Ammonia and greenhouse gases emissions from cow's excreta are affected by feeding system, stage of lactation and sampling time

Table S5. Effect of sampling time on faecal and urinary characteristics.

	Sampling times			SEM	<i>p</i>
	T8h	T12h	T17h		
<i>Faeces</i>					
pH	6.88	6.84	7.06	0.05	0.073
Dry matter (%)	13.6	13.9	13.5	0.17	0.328
Total C (%)	39.9	40.6	40.4	0.18	0.095
Organic soluble C (%)	0.13	0.14	0.13	0.01	0.459
Total N (%)	2.35	2.38	2.23	0.05	0.206
Ammoniacal-N (mg/kg)	200 ^{ab}	248 ^a	169 ^b	14.3	0.043
Nitric-N (mg/kg)	380	319	383	54.31	0.671
Organic soluble N (%)	0.04	0.03	0.12	0.05	0.406
<i>Urine</i>					
pH	8.56	8.50	8.52	0.02	0.145
Dry matter (%)	5.8	5.81	5.06	0.30	0.238
Total C (%)	1.30	1.38	1.17	0.08	0.254
Total N (%)	0.71	0.78	0.61	0.04	0.077
Ammoniacal-N (mg/L)	582	562	454	38.0	0.145
Ureic-N (%)	0.33	0.34	0.30	0.02	0.457
Organic soluble N (%)	0.65	0.72	0.57	0.03	0.079

T8h - sampling time before a.m. milking; T12h - sampling time at middle day; T17h - sampling time before p.m. milking; SEM – Standard error of the mean. Values followed by different lowercase superscript letters in rows indicate significantly different means ($p < 0.05$).

Ammonia and greenhouse gases emissions from cow's excreta are affected by feeding system, stage of lactation and sampling time

Table S6. Effect of the interaction between stage of lactation and sampling time on faecal and urinary characteristics.

	Peak			Post peak			SEM	<i>p</i>
	T8h	T12h	T17h	T8h	T12h	T17h		
<i>Faeces</i>								
pH	6.90	6.90	7.19	6.86	6.78	6.93	0.07	0.394
Dry matter (%)	13.5	13.6	13.6	13.7	14.3	13.5	0.24	0.321
Total C (%)	40.1	40.6	40.1	39.6	40.7	40.6	0.26	0.321
Organic soluble C (%)	0.13	0.13	0.11	0.13	0.15	0.14	0.01	0.613
Total N (%)	2.33	2.48	2.31	2.37	2.29	2.15	0.07	0.332
Ammoniacal-N (mg/kg)	195	260	173	205	253	165	20.2	0.722
Nitric-N (mg/kg)	353	293	378	407	346	388	76.8	0.949
Organic soluble N (%)	0.06	0.03	0.03	0.03	0.02	0.20	0.06	0.326
<i>Urine</i>								
pH	8.57	8.49	8.50	8.56	8.50	8.53	0.03	0.746
Dry matter (%)	6.02	5.94	4.98	5.57	5.68	5.15	0.42	0.765
Total C (%)	1.43	1.47	1.17	1.17	1.30	1.17	0.11	0.530
Total N (%)	0.76	0.82	0.59	0.65	0.73	0.63	0.05	0.425
Ammoniacal-N (mg/L)	650	617	379	513	508	529	53.8	0.099
Ureic-N (%)	0.32	0.34	0.27	0.34	0.34	0.33	0.03	0.618
Organic soluble N (%)	0.70	0.76	0.56	0.60	0.68	0.58	0.05	0.513

T8h - sampling time before a.m. milking; T12h - sampling time at middle day; T17h - sampling time before p.m. milking; SEM – Standard error of the mean.

Chapter Five

Implications of seasonal and daily variation on methane and ammonia emissions from naturally ventilated dairy cattle barns in a Mediterranean climate: A two-year study

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Abstract

Seasonal and daily variations of gaseous emissions from naturally ventilated dairy cattle barns are important figures for the establishment of effective and specific mitigation plans. The present study aimed to measure CH₄ and NH₃ emissions in three naturally ventilated dairy cattle barns covering the four seasons for two consecutive years. In each barn, air samples from five indoor locations were drawn by a multipoint sampler to a photoacoustic infrared multigas monitor, along with temperature and relative humidity. Milk production data were also recorded. Results showed seasonal differences for CH₄ and NH₃ emissions in the three barns with no clear trends within years. Globally, diel CH₄ emissions increased in the daytime with high intra-hour variability. The average hourly CH₄ emissions (g h⁻¹ livestock unit⁻¹ (LU)) varied from 8.1 to 11.2 and 6.2 to 20.3 in the dairy barn 1, from 10.1 to 31.4 and 10.9 to 22.8 in the dairy barn 2, and from 1.5 to 8.2 and 13.1 to 22.1 in the dairy barn 3, respectively, in years 1 and 2. Diel NH₃ emissions highly varied within hours and increased in the daytime. The average hourly NH₃ emissions (g h⁻¹ LU⁻¹) varied from 0.78 to 1.56 and 0.50 to 1.38 in the dairy barn 1, from 1.04 to 3.40 and 0.93 to 1.98 in the dairy barn 2, and from 0.66 to 1.32 and 1.67 to 1.73 in the dairy barn 3, respectively, in years 1 and 2. Moreover, the emission factors of CH₄ and NH₃ were 309.5 and 30.6 (g day⁻¹LU⁻¹), respectively, for naturally ventilated dairy cattle barns. Overall, this study provided a detailed characterization of seasonal and daily gaseous emissions variations highlighting the need for future longitudinal emission studies and identifying an opportunity to better adequate the existing mitigation strategies according to season and daytime.

Keywords: Ammonia; Dairy barns; Emission factors; Methane; Natural ventilation; Seasonality

Introduction

The mutual effect between agriculture and climate change requires actions to mitigate the negative impacts of the first, and adaption measures to improve the sector's resilience to climate risks to suppress the demand for a projected worldwide population growth of 9.7 billion by 2050 [1, 2]. Milk and dairy products are essentials in a balanced diet playing an important role in food security, being the reduction of environmental footprint, particularly of CH₄ and NH₃ emissions, a priority for the dairy cattle sector [3].

Methane is the second largest GHG in terms of radiative forcing, with a GWP of 28 in relation to CO₂ [4], and is a precursor to tropospheric ozone, a harmful pollutant for human, crop production and ecosystems [5]. According to EEA [6]), enteric fermentation is responsible for 81% of the total CH₄ emissions from the agriculture sector, with dairy cows contributing nearly 40%. Enteric CH₄ is primarily formed in the rumen by methanogens during microbial fermentation of fibrous feedstuffs, being mainly released by eructation and representing 2–12% loss of feed energy for the cow [7]. Its production varies with diet composition, breed, and lactation stage, among others [8-10].

Manure management including housing, storage, and application to croplands accounts for the great majority of NH₃ from the livestock sector [11]. At the animals' barn floor, the urinary urea is hydrolyzed by fecal urease activity, forming NH₄⁺/NH₃. The NH₃ volatilization process is correlated with pH, air temperature, and velocity, driving the mass transfer process of NH₃ from liquid manure to the surface and the air [12, 13]. Additionally, N₂O can also be formed during incomplete nitrification/denitrification processes of NH₃ into N₂ [14]. Moreover, atmospheric NH₃ is the main source of NH₄⁺ (an aerosol constituent), the precursor of secondary particles (components of PM_{2.5}), and upon deposition, can cause severe impacts on human health and the environment [15, 16].

Gaseous emissions vary among dairy cattle barns due to architectural characteristics, feeding and manure management systems, and climate parameters, among others [17, 18]. Measurements of gaseous emissions are not only crucial to derive country-specific emission factors (EFs) and to characterize emissions at different levels but also to allow the definition of appropriate mitigation strategies [19, 20]. Several European countries have been monitoring GHG and NH₃ emissions from naturally ventilated dairy cattle barns as Germany [21-24], Italy [25], Poland [26, 27], Denmark [28, 29], and Sweden [30, 31], while in many others, including in Portugal, no available published data are reporting on-farm GHG emission from naturally ventilated dairy cattle barns.

In this context, the current work aimed to measure CH₄ and NH₃ emissions from three naturally ventilated dairy cows' barns, and to identify seasonal and daily gaseous emissions variations. We hypothesize that the outcomes of this work will contribute data to

improve the Mediterranean and European EFs of CH₄ and NH₃ for dairy cattle barns in the future.

Material and Methods

Measurement sites

Measurements were conducted over two years in three dairy cattle barns located at Vila do Conde, in the Northwest (NW) of Portugal. All three barns were of free stall type housing with cubicles and naturally ventilated by large openings on the side walls, open ridges on the roof, and ceiling fans ensuring the air re-circulation. The free-stall zones had solid concrete floors and a cubicle system with bedding sawdust in the lying area. In all barns, the manure handling system equipped with an automatic scraper removed the manure from the inside buildings to the outside manure pit. The detailed characterization of the dairy barns is presented below, and the dairy effective is described in Table 12.

Table 12. Description of cow's number and milk production parameters of dairy farms in the respective measurement period.

		Date		Number of cows			Milk yield (kg/cow/day)			Days in milk		
Year	Season	Start	End	Farm 1	Farm 2	Farm 3	Farm 1	Farm 2	Farm 3	Farm 1	Farm 2	Farm 3
1	Winter	07/12/2016	26/01/2017	162	95	60	34.6	37.8	31.4	157	160	190
1	Spring	10/03/2017	18/04/2017	158	99	61	34.7	37.8	33.3	177	178	182
1	Summer	13/07/2017	26/08/2017	159	97	64	32.4	37.2	32.4	194	183	195
1	Autumn	25/10/2017	21/11/2017	162	100	65	34.6	39.0	30.8	157	162	183
2	Winter	22/12/2017	19/01/2018	165	101	71	35.1	38.8	32.0	144	165	173
2	Spring	14/04/2018	01/05/2018	167	101	70	36.4	38.6	32.5	158	168	186
2	Summer	14/08/2018	11/09/2018	160	101	71	31.2	37.7	31.2	178	160	189
2	Autumn	18/10/2018	17/11/2018	164	101	69	33.2	37.1	32.0	164	162	173

The first dairy cattle barn (dairy barn 1) had approximately 846 m² with 3.60 m eave height and 5.07 m ridge height, and Northeast-Southwest (NE-SW) orientation (Figure 10). The NW side had large openings, the NE and SW sides open doors, and the Este River was located at approximately 50 m of the NW side. The ceiling fans were scheduled to work at temperatures above 24°C. The barn floor was cleaned every 3 h in the feed alley, and every 1 h in the remaining area to an outside manure pit with southern exposure, connected to a manure lagoon on the North (Figure 10). The animals were milked at a milking parlor and fed a total mixed ration twice per day, during early morning and late afternoon.

The second cattle barn (dairy barn 2) had approximately 1600 m² with 4.38 m eave height and 6.59 m ridge height, and NE-SW orientation (Figure 10). The NW side presented large openings with an open door on the S side and a passage to other cattle barn on the SW side. The ceiling fans were scheduled to work at temperatures above 25 °C. The manure scrap system removed the manure floor every 2 h to two concrete manure pits, one on the Southern side and another on the NW side of the barn. The animals were milked with a robotic milking system with customized frequency for each cow, and fed with a total mixed ration twice per day, during early morning and late afternoon, supplemented with a concentrate at the milking robot.

The third cows' barn (dairy barn 3) had approximately 960 m² with 4.38 m and 4.92 m eave height in the Southern hall and the Northern hall, respectively, and 7.13 m ridge height, and with East-West (E-W) orientation (Figure 10). The southern side was closed by a grid and the northern side presented large openings, with ceiling fans schedule to work at temperatures above 22 °C. The manure scrap system removed the manure every 3 h to a cement manure pit located on the W side of the barn. The animals were milked at a milking parlor and fed a total mixed ration delivered once per day, during the late afternoon.

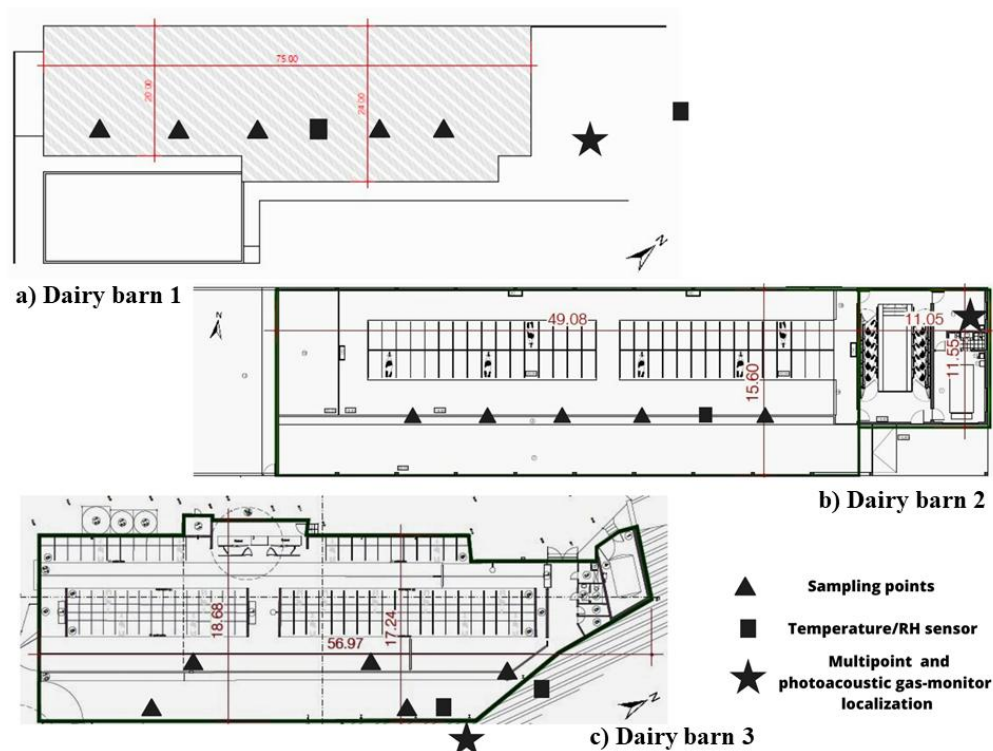


Figure 10 - Schematic representation of the three cattle barns.

Measurement periods and sampling methodology

Measurements of CO₂, CH₄, and NH₃ concentrations covered all seasons of two consecutive years, 2016/2017 (year 1) and 2017/2018 (year 2). The measurement periods of each season, the number of animals in the barn, the milk yield (kg day⁻¹), and average days in milk are presented in Table 13. In each measurement period the diet was sampled and chemically analyzed (Tables S1 to S3).

Gas concentrations were measured in five indoor points at each dairy barn, by a Teflon tubes system (3 mm inner diameter) equipped with a polytetrafluoroethylene membrane (1.0 µm pore size, Whatman, Ome, Japan), to protect them from dust. Sampling points were 2.5 m above the floor and located on the opposite wall of the feed alley in dairy barn 1 and above the feed alley in dairy barns 2 and 3. The air samples were collected at intervals of 2 min per sampling point by a multipoint (model INNOVA 1409–12, Lumasense Technologies, Ballerup, Denmark) connected to a photoacoustic gas-monitor (model INNOVA 1412i-5, Lumasense Technologies). The gas analyzer was calibrated by the manufacturer before the beginning of the study, being operated in a mode for compensation of water interference and cross-interference and equipped with an optical filter for water vapor (filter type SB0527, Lumasense Technologies) and the detection limits for NH₃ (filter type UA0973, Lumasense Technologies), CO₂ (filter type UA0982, Lumasense Technologies).

Technologies) and CH₄ (filter type UA0969, Lumasense Technologies) were, respectively, 152.1, 2947.1 and 286.4 µg m⁻³.

For each experimental date, indoor and outdoor temperatures (°C) and relative humidity (% RH) were assessed in 10 min intervals using sensors (CS215, Campbell Scientific, Leicestershire, UK) and recorded in a micrologger (CR3000, Campbell Scientific).

Data pre-processing

Data pre-processing methodology applied to gases concentration, temperature, and RH time series included three steps: i) data preparation; ii) missing data identification; and iii) missing data interpolation.

The gases concentrations recorded from the five sampling points were hourly averaged, and hourly missing data was interpolated using the Seasonally Splitted Missing Values Imputation model associated with Kalman filtering smoothing. The missing data of indoor temperature was firstly interpolated for 10 min intervals following the same methodology described above, and subsequently hourly averaged.

The gaseous concentrations and indoor temperature were used for emission calculation and were analyzed on a 24 h basis as described in Section 2.4.

Ventilation rate and gases emission calculation methodology

The ventilation rate and gaseous emission were calculated according to the guidelines established by CIGR [32]. The hourly CO₂, CH₄, and NH₃ concentrations from the five indoor points were averaged to represent the indoor concentration in each dairy cows barn, following the methodology described in several research works [22-24].

The ventilation rate (Q, m³/h) was calculated by the indirect CO₂ mass balance method as follows in Equation (1):

$$Q = \frac{CO_{2\text{ prod}}}{(CO_{2\text{ inside}} - CO_{2\text{ outside}})} \times N \quad (1)$$

where CO_{2 prod} is the estimated CO₂ production rate per animal (g/h), N is the animal number in the barn (Table 1), CO_{2 inside} and CO_{2 outside} the average CO₂ concentration of the indoor and outdoor gas (g/m³), respectively (CO_{2 outside} = 0.80 g/m³), [33].

The CO₂ production rate excreted is related to the heat produced (Heat_{prod}) per cow depending on several factors, from feed intake or animal activity to the housing type [32]. Thus, the CO₂ production rate was calculated using the Equation (2):

$$CO_{2\ prod} = \frac{0.185 \times Heat_{prod} \times A}{1000} \quad (2)$$

where $Heat_{prod}$ is multiplied by the relative animal activity (A) and by the 0.185 factor. The $Heat_{prod}$ is calculated from the multiplication of the total heat production (ϕt) by the corrected factor for the temperature inside the building (CF), as follows in Equations (3), (4), (5), (6):

$$Heat_{prod} = \phi t \times CF \quad (3)$$

$$\phi t = 5.6 \times (m)^{0.75} + 1.6 \times 10^{-5} \times (p)^3 + 22y \quad (4)$$

$$CF = 1000 \times (4 \times 10^{-5}(20 - T_i)^3 + 1) \quad (5)$$

$$A = 1 - a \times \sin [(2 \times \pi/24) \cdot (h + 6 - h_{min})] \quad (6)$$

where variable m in Equation (4) represents the average mass of the cow (kg/cow), p the days after insemination (days), and y the milk yield (kg/day, Table 1). The variable A Equation (6) follows the sinusoidal dromedary model for diurnal variation [32]. The constant a expresses the amplitude concerning the constant 1, and h_{min} indicates the minimum activity of the day (hours after midnight).

Following CIGR [32] parameters for dairy cows in cubicles, our study considered $a = 0.22$, and $h_{min} = 2.9$, for the three dairy cattle barns.

The emission rate (E_t , g/h) was calculated as followed in Equation (7):

$$E_t = Q \times (C_i - C_o) \quad (7)$$

where Q (m^3/h) is the ventilation rate calculated Equation (1), and C_i and C_o (g/m^3) are the average indoor and outdoor concentrations, respectively. Cows numbers were expressed in livestock unit (LU), considering that one dairy cow was 1.2 LU [34], with the emission rate converted to LU calculated following Equation (8):

$$E = \frac{E_t \times 1.2}{N \times m} \quad (8)$$

Statistical analysis

Statistical analysis was performed using the R software version 4.0.5 (The R foundation for statistical computing). The model included the fixed effects of barn, season, and year, and the random residual error. The experimental unit was the hourly average of the five sampling points. The Kruskal-Wallis test was employed to compare the seasons in each dairy barn and the years of measurement, and Dunn's *post hoc* test was used for the

respective multiple comparisons ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ were the adopted levels of significance). In this analysis, inter quartile range (IQR) filtering was applied to identify and remove outliers in the dataset [35]. This method involves identifying data points that fall below $Q1 - 1.5 \times IQR$ or above $Q3 + 1.5 \times IQR$, where $Q1$ is the first quartile and $Q3$ is the third quartile of the data distribution.

Results

Gas concentrations, ventilation rate and climate parameters

Average daily CH_4 concentrations varied among dairy barns between years and seasons (Figure 11, Table S10). Indeed, daily CH_4 concentrations (g m^{-3}) ranged from 0.001 to 0.030 and from 0.002 and 0.038 in years 1 and 2, respectively, with dairy barn 3 differing from the others in year 1 ($p < 0.001$), and dairy barn 2 differing from barns 1 and 3 in year 2 ($p < 0.05$, Table S4, Figure 11). Significant differences were observed between autumn and summer ($p < 0.001$) and winter and summer ($p < 0.001$) in dairy barn 1, between summer and winter ($p < 0.01$) and autumn and summer ($p < 0.001$) in dairy barn 2, and between autumn and spring ($p < 0.001$) and autumn and summer ($p < 0.001$) in dairy barn 3, in years 1 and 2, respectively (Table S11 and Table S12).

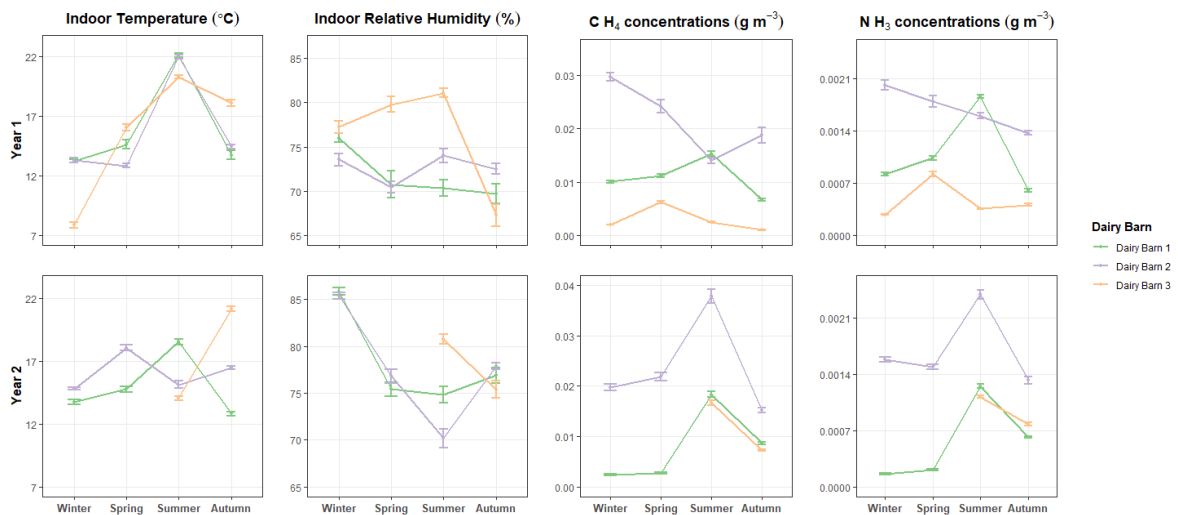


Figure 11 - Environmental parameters and daily average CH_4 and NH_3 concentrations in all barns, in year 1 and year 2. Vertical bars represent the standard error of the mean. CH_4 , methane and NH_3 , ammonia.

Average daily NH_3 concentrations varied among dairy barns between years and seasons (Figure 11). In fact, daily NH_3 concentrations (g m^{-3}) varied from 2.8×10^{-5} to 0.0020 and from 0.0002 to 0.0024 in years 1 and 2, with dairy barn 3 differing from the others ($p < 0.001$), and barn 1 differing from barn 2 in year 1 ($p < 0.05$), and dairy barn 2 differing from barns 1 and 3 in year 2 ($p < 0.001$ and $p < 0.01$, respectively, Table S10,

Figure 11). Significant differences were observed between winter and summer ($p < 0.05$) and summer and autumn ($p < 0.001$) in dairy barn 1, between winter and spring ($p < 0.001$), winter and autumn ($p < 0.05$) and spring and summer ($p < 0.01$) in dairy barn 3 in year 1 (Table S11). Moreover, between winter and summer ($p < 0.001$), winter and autumn ($p < 0.05$), and spring and summer ($p < 0.001$) in dairy barn 1, between winter and summer ($p < 0.05$), spring and summer ($p < 0.01$), and summer and autumn ($p < 0.001$) in dairy barn 2, and between summer and autumn ($p < 0.01$) in dairy barn 3, in year 2 (Table S12).

Average daily indoor temperatures were similar between dairy barns in year 1 ($p > 0.05$) and in year 2 dairy barn 1 differed from 2 ($p < 0.05$ Table S13, Figure 11). Besides, ranged from 13°C to 22°C and 12°C to 18°C in dairy barn 1 (winter and summer, $p < 0.001$, and autumn and summer, $p < 0.01$), from 13°C to 22°C and 15°C to 18°C in dairy barn 2 (spring and summer, $p < 0.001$, and winter and spring, $p < 0.01$, respectively), and from 8°C to 20°C and 14°C to 21°C in dairy barn 3 (winter and summer, $p < 0.001$, and summer and autumn, $p < 0.001$), during year 1 and 2, respectively (Figure 11, Table S14 and Table S15). Moreover, indoor temperature differed between summer and autumn in dairy barns 1 and 2 ($p < 0.001$ and $p < 0.01$, respectively), and winter and autumn in dairy barn 3 ($p < 0.01$) during year 1 (Table S14).

Average daily indoor RH was similar between dairy barns in years 1 and 2 ($p > 0.05$, Table S13). Differences in indoor RH in year 1 were only identified in dairy barn 3 between summer and autumn ($p < 0.05$, Table S14), while during year 2 varied from 75% in spring to 86% in winter in dairy barn 1 ($p < 0.01$), 70% in summer to 85% in winter in dairy barn 2 ($p < 0.001$), 75% in autumn to 81% in summer in dairy barn 3 ($p < 0.05$, Table S15, Figure 11).

The average daily ventilation rate in the dairy barn 3 differed from dairy barns 1 and 2, in year 1, while dairy barn 2 differed from dairy barns 1 and 3, in year 2 ($p < 0.001$, Table S4, Figure 12). Besides, hourly ventilation ranged from 830 to 1258 g h⁻¹ LU⁻¹ in the dairy barn 1 (summer and autumn, $p < 0.001$), 479 to 2543 g h⁻¹ LU⁻¹ in the dairy barn 2 (winter and autumn, $p < 0.001$), and 1513 to 3475 g h⁻¹ LU⁻¹ in the dairy barn 3 (spring and winter, $p < 0.001$, Table S5) in year 1 (Figure 12). In dairy barn 1, ventilation rate differed among seasons, varying from 7101 in winter to 1007 g h⁻¹ LU⁻¹ in summer ($p < 0.001$) and to 1878 g h⁻¹ LU⁻¹ in autumn ($p < 0.01$), in year 2 (Table S11 and Table S12).

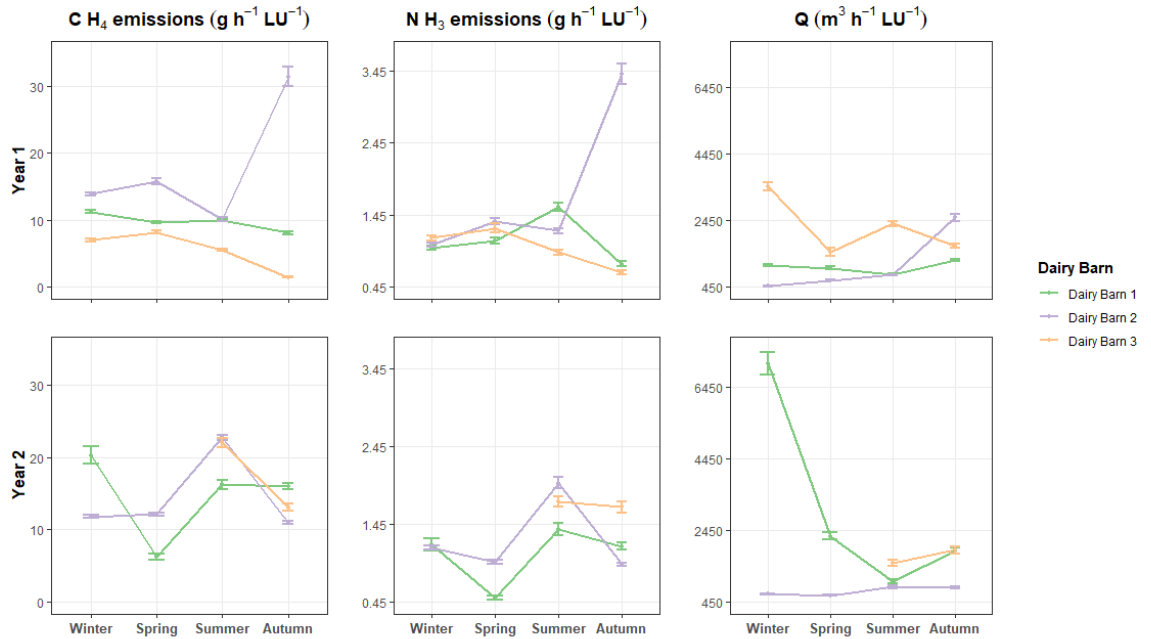


Figure 12 - Hourly average CH₄ and NH₃ emissions and ventilation rate in all barns, in year 1 and year 2. Vertical bars represent the standard error of the mean. CH₄, methane, NH₃, ammonia and, Q, ventilation rate.

Daily patterns and 24-hour profile of methane emissions

Generally, average hourly CH₄ emissions in years 1 and 2 were lower than 25 g h⁻¹ LU⁻¹, except in the autumn of year 1 at barn 2 that was higher than 30 g h⁻¹ LU⁻¹. Diel CH₄ emissions increased during the daytime and presented high intra hour variability from 9 a.m. to 7 p.m. (Figure 13). This pattern is particularly marked in the winter and autumn of years 1 and 2, in the spring of year 1 and summer of year 2, nonetheless in the autumn of year 1 in dairy barn 3 emissions followed the opposite pattern (Figure 13). In more detail, average hourly CH₄ emissions were significantly different among all dairy barns in year 1 contrarily to year 2 (Table S16, Figure 13). Besides, hourly CH₄ emissions varied from 8.1 to 11.2 g h⁻¹ LU⁻¹ and 6.2 to 20.3 g h⁻¹ LU⁻¹ in dairy barn 1 (autumn and winter, $p < 0.001$ and spring and winter, $p > 0.05$), from 10.1 to 31.4 g h⁻¹ LU⁻¹ and 10.9 to 22.8 g h⁻¹ LU⁻¹ in dairy barn 2 (summer and autumn, $p < 0.001$, autumn and summer, $p < 0.001$), and from 1.5 to 8.2 and 13.1 to 22.1 g h⁻¹ LU⁻¹ in dairy barn 3 (autumn and spring, $p < 0.001$, and autumn and summer, $p < 0.001$), in years 1 and 2, respectively (Figure S1, Tables S11 and S12).

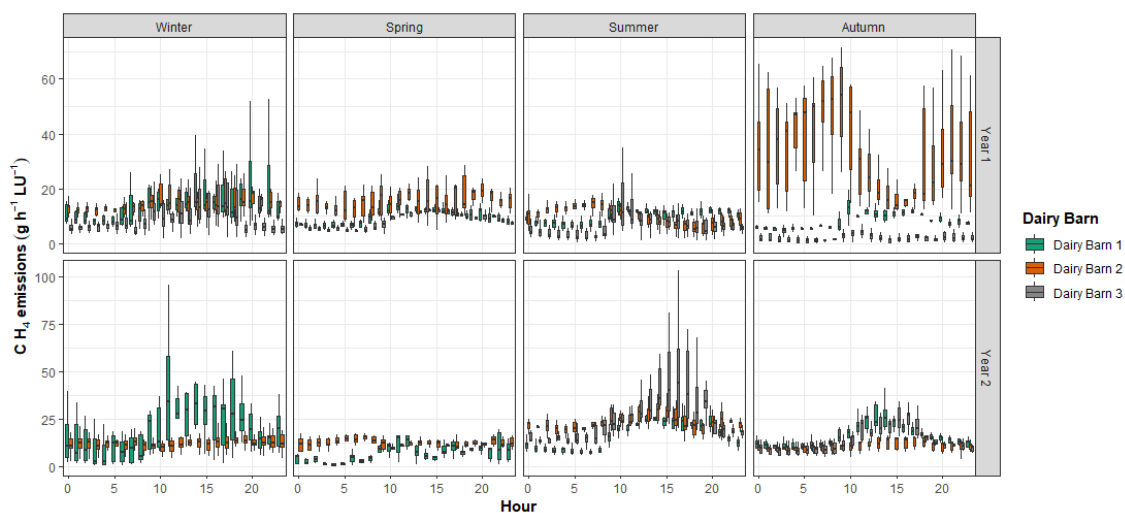


Figure 13 - Diel variations of CH₄ emissions in all barns, in year 1 and year 2.

Furthermore, daily CH₄ emissions presented seasonal intraday variation in dairy barns (Figure S1). In dairy barn 1, daily variability was low in all seasons, except in the first days of winter in year 1, while in year 2 the winter season presented the highest daily variability followed by summer (Figure S1). In dairy barn 2, the winter season showed the highest daily variability, contrary to autumn and spring in year 1, while in year 2 daily variability was globally low (Figure S1). In dairy barn 3, the daily variability was low in year 1, particularly in the autumn season, with some exceptions in specific days of winter and summer (Figure S1).

Daily patterns and 24-hour profile of ammonia emissions

Globally, average hourly NH₃ emissions in years 1 and 2 were lower than 2.45 g h⁻¹ LU⁻¹, except in the autumn of year 1 at barn 2. Diel NH₃ emissions were consistently higher with high variability during the daytime (8 a.m. to 8 p.m.) compared to the nighttime, which showed lower variability in most seasons.

In particular, average hourly NH₃ emissions in dairy barn 2 differed from 3 ($p < 0.001$), and barn 1 from barn 3 in year 2 ($p < 0.05$ Table S10). Moreover, hourly NH₃ emissions varied from 0.77 to 1.56 g h⁻¹ LU⁻¹ and from 0.50 to 1.38 g h⁻¹ LU⁻¹ in dairy barn 1 (autumn and summer, $p < 0.001$ and spring and summer $p < 0.01$), from 1.04 to 3.40 g h⁻¹ LU⁻¹ and from 0.93 and 1.98 g h⁻¹ LU⁻¹ in dairy barn 2 (winter and autumn, $p < 0.001$, and autumn and summer $p < 0.001$), from 0.66 to 1.32 g h⁻¹ LU⁻¹ and 1.67 and 1.73 g h⁻¹ LU⁻¹ in dairy barn 3 (autumn and winter, $p < 0.001$, and autumn and summer, $p > 0.05$), in year 1 and year 2, respectively (Tables S11 and S12).

Daily NH₃ emissions variability in dairy barn 1 was higher at the beginning of the winter and autumn when compared to the remaining seasons. In year 2 daily variability was

globally greater, with the highest emissions identified in the winter and summer (Figure S2). In dairy barn 2, the winter season presented the highest daily variability and summer the lowest in year 1, while the daily variability was higher in summer and lower in the autumn in year 2 (Figure S2). In dairy barn 3, daily variability differed within each season of year 1 and was higher in the summer compared to autumn in year 2 (Figure S2).

Besides, diel NH_3 emissions were particularly high and with high variability during the daytime (8 a.m. to 8 p.m.) in the winter in dairy barns 1 and 2 (years 1 and 2), in the spring in barns 1, 2, and 3 (year 1), and barn 2 (year 2), in the summer in barn 3 (year 1), and barn 2 and 3 (year 2) and in the autumn in dairy barn 2 (years 1 and 2) (Figure 14). Additionally, NH_3 emissions in the autumn in the dairy barn 2 presented the highest and greater variability compared to the remain measurement periods in year 1 (Figure S2).

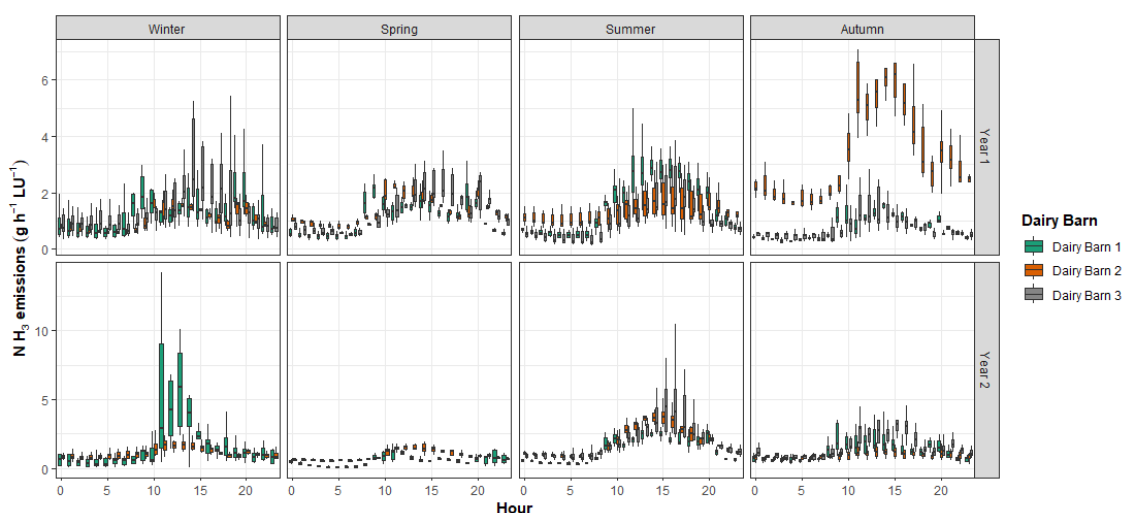


Figure 14 - Diel variations of NH_3 emissions in all barns, in year 1 and year 2.

Discussion

Seasonal and daily variation of CH_4 emissions

The three dairy cattle barns studied had a solid concrete floors, the manure pit was only located outside, and floor cleaning frequency ranged between 1 and 3 h in all barns, suggesting that the great majority of CH_4 measured in this study was from enteric fermentation [36]. Indeed, the anaerobic degradation of manure stored in manure pits located underneath animals barn or outside were estimated to represent 24% of total CH_4 emissions from livestock [37]. Besides, the barns were naturally ventilated by large open windows and passages, leading to the greater influence of the outdoor climate parameters on the indoor conditions. In our study, daily CH_4 emissions varied among seasons similar to Tabase *et al.* [38], while Saha *et al.* [22] only found the influence of one season (autumn) in CH_4 emissions. Interestingly, Cortus *et al.* [39] and Huang and Guo [40] identified

variations of CH₄ concentrations among seasons, however with no variation in CH₄ emissions. A possible explanation for the contrasting results observed may be the sliding windows, the curtains, and the high density of fans of the barns studied by these authors, ensuring stability of indoor parameters and constant ventilation.

Diet composition strongly modulates CH₄ production from enteric fermentation, with forage-based diets known to promote acetate whereas starch-rich diets favor propionate production, which provides a sink for H₂, resulting in less CH₄ emission in the rumen [41]. In our study, although the chemical composition of diets was similar among dairy barns, dairy barn 2 differed from the other barns by including alfalfa in the cows' diet. Despite this difference, no effect on CH₄ emissions was detected. Thus, our results suggest an opportunity to increase farm self-sufficiency using this high-quality protein source, with no detrimental effects on CH₄ emissions [42]. Besides, genetics, animal activity, and climate parameters such as ambient temperature and RH can cause stress in dairy cows and account for the changes in dry matter intake, drinking frequency, feed efficiency, and rumination time with consequent reduction in milk production and affecting CH₄ emission patterns, not only seasonally but also daily [43, 44].

In general, the results of this study showed higher diel CH₄ emissions during daytime similar to Saha *et al.* [22]) and Joo *et al.* [45]), with higher intra hour variability from 9 a.m. to 7 p.m. in all seasons, maybe due to the greater animal activity during the daytime compared to nighttime [36]. Moreover, an increase of CH₄ emissions up to 2 h after feeding followed by a decrease in the values until the next meal has been described in the literature [46-48]. In this study, this pattern was not identified in all measurements, despite the different feeding systems of the studied dairy barns. Moreover, diel CH₄ emissions increased in the early morning for dairy barns 1 and 2, roughly matching the first (of two) feed distributions; dairy barn 3, which only had one feed distribution (at late afternoon), presented the same behavior. Despite the differences in feeding distribution, the chemical composition of the cow's diet was similar among the three dairy barns studied in all periods. Thus, these results may be more related to cow's feeding behavior than to differences in feeding distribution as proposed by Muñoz-Tamayo *et al.* [49]). During the daytime is also possible to identify smaller CH₄ emissions peaks in all dairy barns, except during the spring of year 1 in dairy barns 1 and 3. A larger second CH₄ emission peak in late afternoon was detected only in the winter of years 1 and 2 in the dairy barn 1, and the spring and autumn of year 1 in the dairy barn 2. In the other seasons, the CH₄ emissions decreased at nighttime, however in dairy barn 2 the emissions decreased slower when compared to dairy barns 1 and 3 possibly due to concentrate delivering in robot milking.

Feeding rate and time spent eating is expected to be higher after diet delivery, however, despite one feed delivery in late afternoon, CH₄ emissions in the dairy barn 3

increased during the daytime, following a similar pattern to dairy barn 2, highlighting the higher animal's activity during the daytime compared to nighttime [30].

Seasonal and daily variation of NH₃ emissions

The correlation between NH₃ emissions and temperature has been described in several studies as positive [17, 50, 51] or negative [38, 52]. Similarly, the correlation between NH₃ emissions and RH is also still uncertain being described as positive [38] and negative [22, 50, 52].

In dairy barn 1 in year 1, the average NH₃ emissions were different between summer and autumn, as well as indoor temperature while RH did not differ significantly. The same behavior was observed in the dairy barn 2 between summer and autumn, and in the dairy barn 3 between winter and autumn, in year 1.

Manure decomposition in the floor is the main emission source of NH₃ from dairy cattle barns, particularly the hydrolysis of the urinary-urea by urease enzyme produced from fecal bacteria, whose activity depends on pH, temperature, urinary urea content, and barn floor characteristics [53, 54]. Although the results of the present study suggest the effect of temperature and RH on NH₃ emissions in some seasons, it also highlights the impact of other parameters on NH₃ emissions. Significant differences between seasons were identified in indoor temperature but were not identified in NH₃ emissions, such as in dairy barn 1 in winter and summer for both years, and in dairy barn 2 in winter and summer and winter and autumn, or in dairy barn 3 in winter and summer and summer and autumn, in years 1 and 2, respectively.

Furthermore, the daily variation in NH₃ emissions showed consistency, increasing with high variability during the daytime, from 8 a.m. to 8 p.m. Daily variances in NH₃ emissions from ventilated dairy barns were also described in the study of Saha *et al.* [22] and of Huang and Guo [55]. It is also possible to identify in this study, for all periods, a peak in the middle of the day, with small variations on the specific hour of said peak among the different seasons. The highest outdoor temperature in the middle of the day may be an additional explanation for this emission peak. Contrarily to the present study, D'Urso *et al.* [18] found two distinct emission peaks, the first during cleaning between 7 a.m. and 8 a.m. and the second after milking. Interestingly, in this study, the three dairy cows' barns studied differed in the milking system; however, all presented the same consistent emission peak. These results are in line with the findings of Rodrigues *et al.* [56], who described higher cumulative N-NH₃ emissions (g kg⁻¹ excreta, total N, and organic soluble N) at 12 h when compared to 8 h and 17 h. Therefore, a possible higher urease activity occurred in the middle of the day, with excreta characteristics favoring volatilization of NH₃.

Emission factors and strategies to mitigate CH₄ and NH₃ emissions from dairy cattle barns

The IPCC provides guidance on methods to estimate emissions from livestock [57]. Thus, many studies have been conducted to enhance the national emission inventories in different countries. Besides, databases can be established to compile EFs from different inventories to a more comprehensive understanding of variations among animal categories, livestock barns, and regions, among others [58]. Considering the seasonal and daily variation of CH₄ and NH₃ emissions previously reported in naturally ventilated dairy cattle barns, the present study suggests that national EFs should be derived from *in loco* campaigns and include correction factors for accurate assessment of climate, animal, and housing variables.

The EF of CH₄ observed in this study was 309.5 g/day/LU, which was in line with the results reported in the literature for dairy cattle barns (Table 13). The EF of CH₄ reported in Central Europe were between 283.0 and 330.0 g/day/LU [22, 24, 26], in northern Europe between 205.7 and 304.0 g/day/LU [28, 59], and Canada between 259.2 and 313.2 g/day/LU [36, 55]. Despite some research works varied in the measurement periods making comparisons between studies difficult, the differences among EFs can be mainly related to climate conditions and the architectural characteristics of cattle barns [37, 55]

Table 13. Selected literature values of average NH₃ and CH₄ emission factors from dairy cattle barns.

Country	Measurements	Emission factors (g/day/LU)		Reference
	Seasons	CH ₄	NH ₃	
Portugal	All	309.5	30.6	Present study
Germany	All	308.4	-	[24]
Canada -	All	259.2	-	[55]
Poland	Spring, summer & autumn	283.2	19.0	[26]
USA-Washington	Spring, autumn & winter	-	21.6	[45]
Denmark	Winter & summer	205.7	12.2	[59]
Germany	All	330.2	56.2	[22]
Switzerland	All	-	36.4	[60]
Denmark	Spring, autumn & winter	304.9	80.0	[28]
Portugal	All	-	43.7	[61]

There are several effective strategies developed to reduce CH₄ emissions from dairy cattle barns, from nutritional to herd management, without impairing animals' performance [14, 63, 64]. Feed additives for enteric CH₄ reduction, such as 3NOP have been intensively studied in the last years, with results reporting a 28 to 32% decrease in CH₄ emissions [65]. However, the length of effectiveness is still the major obstacle to feed additives due to the ruminal microbial resistance development [66]. Besides, the effect of 3-nitrooxypropanol on dry matter intake, rumen fermentation parameters, and microbial composition must be explored in the future [67]. Also, high-quality forages, lower forage to concentrate ratio and increased feeding levels are known approaches to decrease CH₄ emissions [64, 68]. Besides, improvement of barn conditions to control temperature and RH such as higher fan density or sprinklers could reduce heat stress of cows known to affect CH₄ production [69].

Reducing the age at first calving and enhancing animal health also decrease CH₄ emissions [70]. Besides, genetic improvement of traits linked to CH₄ production can also be considered as a long-term mitigation approach towards breeding for low-emitters cows [71]. These available strategies can be applied in the production systems studied in the present research.

The EF of NH₃ in this study was 30.6 g/day/LU which was within the range described in the literature (Table 13). Studies performed in Central Europe reported average NH₃ emissions between 19.0 and 80.0 g/day/LU [22, 26, 61, 62], in northern Europe between 12.2 and 80.0 g/day/LU [28, 59], in United Kingdom of 23.1 g/day/LU [72] and 21.6 g/day/LU in Washington state of United States of America [60].

Different sustainable options are available to minimize NH₃ emissions from livestock such as feeding strategies, floor design, and manure management at the barn, storage, treatment operations, and when applied to soil [53, 73]. A decrease of 1% of the protein content of the animal feed was suggested to reduce up to 15% of the total NH₃ emissions from barn if a balanced amino acid supply is granted [73]. Inclusion of some ingredients (such as sunflower oil or red clover silage) and amino acids supplementation can improve N retention while reducing N emissions [74-76]. Besides, the determination of dairy cow's metabolizable protein requirements, or shift from urine to fecal excretion of N are important strategies used to improve N use efficiency and reduce N emissions [77, 78]. Differences in cow's stages of lactation within dairy barns could be addressed in the future, particularly the manure management in the housing floor, where cumulative N–N₂O emissions (g kg⁻¹ total N, and ammoniacal-N) and cumulative N–NH₃ (g kg⁻¹ excreta, and total N) differed between stages of lactation [56]. The decrease of manure surface area in the barn floor, rapid removal of urine, separation of urine and faeces or urine drainage are recommended techniques to decrease NH₃ emissions from the

animal's barn [73]. Studies on the effect of floor type on NH_3 emissions showed a reduction of 46% with grooved floors and 35% with grooved floors without perforations when compared to slatted floors [80]. Besides, a solid floor with a 3% of inclination and a V-shaped can reduce NH_3 volatilization by 21% and 50%, respectively, when compared to a slatted floor [80]. Moreover, to ensure an optimal barn climatization roof insulation and/or automatically controlled natural ventilation are recommended techniques that can reduce 20% of NH_3 volatilization [73].

Conclusion

The seasonal differences found for CH_4 and NH_3 emissions in the three dairy barns indicate the climate parameter's effect on dairy barns, in which open windows and large openings do not ensure the stability of indoor temperature and RH and constant ventilation among seasons. Diel CH_4 and NH_3 emissions increased during the daytime with higher intra hour variability, when compared to nighttime. This fact may reflect the higher animal activity during the day, being also in this period of the day where mitigation approaches must be mainly focused, such as barn climatization and cleaning frequency.

The EFs for CH_4 and NH_3 were 309.5 and 30.6 g/day/LU, being in line with the literature on the topic and several mitigation strategies can be adopted to reduce gaseous emissions at the barn level. The insight of this study provides a better understanding of daily and seasonal CH_4 and NH_3 emissions variations from naturally ventilated dairy cattle barns. These variations should be further explored in future works and considered in the definition of mitigation plans in the short- and long-term.

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Author contributions

Ana Rodrigues: Investigation; Formal analysis; Writing - original draft; Writing - review & Editing. Maria Silva: Formal analysis; Supervision; Writing - review & Editing. Vanessa Silva: Formal analysis; Writing - review & Editing. Margarida Maia: Writing - review & editing. Ana Cabrita: Writing - review & editing. Henrique Trindade: Conceptualization; Supervision; Writing - review & Editing. António Fonseca: Funding acquisition; Conceptualization; Resources; Supervision; Writing - review & editing. José Pereira: Conceptualization; Resources; Supervision; Writing - review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary material

Table S7. Ingredient (kg as-fed) and chemical composition (g/kg dry matter, DM) of the diets offered in dairy barn 1.

	Year 1				Year 2			
	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
Ingredient composition								
Corn silage	34	34	30	32	35	35	35	37
Barley straw	1.6	2	1	1	1.5	1.5	1.7	1.5
Ryegrass	-	-	6	-	-	-	-	-
Raw material mixture	10	-	-	-	9.5	-	-	-
Concentrate	-	-	-	-	-	-	-	-
Chemical composition								
DM (g/kg)	489	506	485	531	498	489	497	427
CP	168	192	186	165	157	139	163	142
NDF	370	361	353	357	344	344	340	398
Starch	255	288	229	239	279	262	269	226

Diets analyzed according to official methods (AOAC, 1990) for dry matter (DM, method 934.01), Kjeldahl N (method 954.01), crude protein (CP) was calculated as Kjeldahl N $\times$ 6.25, neutral detergent fiber (NDF, with α -amylase and without sodium sulfite, and expressed exclusive of residual ash).

Table S8. Ingredient (kg as-fed) and chemical composition (g/kg dry matter, DM) of the diets offered in dairy barn 2.

	Year 1				Year 2			
	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
Ingredient composition								
Corn silage	33	33	30	32	32	30	28	28
Barley straw	1.6	2	0.4	0.8	0.7	1.2	0.7	0.5
Ryegrass	7	10	4	-	4.5	12	5	5
Alfalfa	-	-	-	-	-	-	-	5
Alfalfa silage	-	-	6	2	-	-	10	-
Raw material mixture	4	4	4	5	5	4	4	4
Concentrate	-	-	-	-	-	-	-	-
Chemical composition								
DM (g/kg)	360	386	419	419	449	461	386	392
CP	139	148	145	145	132	141	146	133
NDF	409	416	378	378	406	392	396	396
Starch	222	207	245	245	197	298	279	252

Diets analyzed according to official methods (AOAC, 1990) for dry matter (DM, method 934.01), Kjeldahl N (method 954.01), crude protein (CP) was calculated as Kjeldahl N $\times$ 6.25, neutral detergent fiber (NDF, with α -amylase and without sodium sulfite, and expressed exclusive of residual ash).

Table S9. Ingredient (kg as-fed) and chemical composition (g/kg dry matter, DM) of the diets offered in dairy barn 3.

	Year 1				Year 2			
	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
Ingredient composition								
Corn silage	32	32	32	29	31	29	30	30
Barley straw	1.7	1.7	1.7	1.5	1.5	1.5	1.5	1.5
Ryegrass silage	3	3	3	3	-	3	3	3
Raw material mixture	9	9	9	10	10	10	10	10
Concentrate	-	-	-	-	-	-	-	-
Chemical composition								
DM (g/kg)	473	495	509	509	507	508	538	455
CP	176	171	188	154	143	152	158	158
NDF	361	359	342	356	338	364	340	358
Starch	223	214	263	264	265	251	268	248

Diets analyzed according to official methods (AOAC, 1990) for dry matter (DM, method 934.01), Kjeldahl N (method 954.01), crude protein (CP) was calculated as Kjeldahl N $\times$ 6.25, neutral detergent fiber (NDF, with α -amylase and without sodium sulfite, and expressed exclusive of residual ash).

Table S10. Comparison of CH₄ and NH₃ concentrations (g/m³) and ventilation rate (Q) between dairy barns during years 1 and 2.

Parameter	Year	Kruskal-Wallis test		Dunn's multiple comparisons test (<i>p</i>)		
		H	<i>p</i>	Farm 1 / Farm 2	Farm 1 / Farm 3	Farm 2 / Farm 3
CH ₄	1	0.697	***	ns	***	***
	2	0.389	**	***	ns	**
NH ₃	1	0.628	***	*	***	***
	2	0.520	***	***	ns	**
Q	1	0.357	***	ns	***	***
	2	0.564	***	***	ns	***

H: H test value, ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Table S11. Comparison of CH₄ and NH₃ concentrations (g/m³) and ventilation rate (Q) between seasons in dairy barns during year 1.

Parameter	Dairy farm	Kruskal-Wallis test		Dunn's multiple comparisons test (<i>p</i>)					
		H	<i>p</i>	Winter / Spring	Winter / Summer	Winter / Autumn	Spring / Summer	Spring / Autumn	Summer / Autumn
CH ₄	1	0.437	***	ns	ns	*	ns	ns	***
	2	0.265	**	ns	**	ns	ns	ns	ns
	3	0.519	***	***	ns	ns	*	***	ns
NH ₃	1	0.484	***	ns	*	ns	ns	ns	***
	2	0.150	*	ns	ns	ns	ns	ns	ns
	3	0.559	***	***	ns	*	**	ns	ns
Q	1	0.323	***	ns	*	ns	ns	ns	***
	2	0.641	***	ns	ns	***	ns	*	ns
	3	0.591	***	***	*	***	ns	ns	ns

H: H test value, ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Table S12. Comparison of CH₄ and NH₃ concentrations (g/m³) and ventilation rate (Q) between seasons in dairy barns during year 2.

Parameter	Dairy farm	Kruskal-Wallis test		Dunn's multiple comparisons test (<i>p</i>)					
		H	<i>p</i>	Winter	/ Winter	/Winter	/Spring	/Spring	/Summer /
				Spring	Summer	Autumn	Summer	Autumn	Autumn
CH ₄	1	0.861	***	ns	***	**	***	ns	ns
	2	0.485	***	ns	**	ns	ns	ns	***
	3	0.735	***	-	-	-	-	-	***
NH ₃	1	0.814	***	ns	***	*	***	ns	ns
	2	0.336	***	ns	*	ns	**	ns	***
	3	0.569	**	-	-	-	-	-	**
Q	1	0.759	***	ns	***	**	ns	ns	ns
	2	0.058	ns	ns	ns	ns	ns	ns	ns
	3	-0.011	ns	-	-	-	-	-	ns

H: H test value, ns: *p* > 0.05, *: *p* < 0.05, **: *p* < 0.01, ***: *p* < 0.001.

Table S13. Comparison of indoor temperature and relative humidity between dairy barns during years 1 and 2.

Parameter	Year	Kruskal-Wallis test		Dunn's multiple comparisons test (<i>p</i>)		
		H	<i>p</i>	Farm 1 / Farm 2	Farm 1 / Farm 3	Farm 2 / Farm 3
Temperature	1	-0.0120	0.844	ns	ns	ns
	2	0.0702	0.207	*	ns	ns
Relative humidity	1	0.0279	ns	ns	ns	ns
	2	-0.0140	ns	ns	ns	ns

H: H test value, ns: *p* > 0.05, *: *p* < 0.05, **: *p* < 0.01, ***: *p* < 0.001.

Table S14. Comparison of indoor temperature and relative humidity between seasons in dairy barns during year 1.

Parameter	Dairy farm	Kruskal-Wallis test		Dunn's multiple comparisons test (<i>p</i>)					
		H	<i>p</i>	Winter	/ Winter	/Winter	/Spring	/Spring	/Summer /
				Spring	Summer	Autumn	Summer	Autumn	Autumn
Temperature	1	0.677	***	ns	***	ns	**	ns	***
	2	0.596	***	ns	***	ns	***	ns	**
	3	0.789	***	ns	***	**	*	ns	ns
Relative humidity	1	0.0120	ns	ns	ns	ns	ns	ns	ns
	2	-0.0254	ns	ns	ns	ns	ns	ns	ns
	3	0.123	*	ns	ns	ns	ns	ns	*

H: H test value, ns: *p* > 0.05, *: *p* < 0.05, **: *p* < 0.01, ***: *p* < 0.001.

Table S15. Comparison of indoor temperature and relative humidity between seasons in dairy barns during year 2.

Parameter	Dairy farm	Kruskal-Wallis test		Dunn's multiple comparisons test (<i>p</i>)					
		H	<i>p</i>	Winter / Spring	Winter / Summer	Winter / Autumn	Spring / Summer	Spring / Autumn	Summer / Autumn
Temperature	1	0.447	***	ns	**	ns	ns	ns	**
	2	0.326	**	**	ns	*	ns	ns	ns
	3	0.735	***	-	-	-	-	-	***
Relative humidity	1	0.334	**	**	*	ns	ns	ns	ns
	2	0.356	***	*	***	ns	ns	ns	ns
	3	0.186	*	-	-	-	-	-	*

H: H test value, ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Table S16. Comparison of CH₄ and NH₃ emissions between dairy barns during years 1 and 2.

Parameter	Year	Kruskal-Wallis test		Dunn's multiple comparisons test (<i>p</i>)		
		H	<i>p</i>	Farm 1 / Farm 2	Farm 1 / Farm 3	Farm 2 / Farm 3
CH ₄	1	0.640	***	**	***	***
	2	0.0331	ns	ns	ns	ns
NH ₃	1	0.119	***	ns	ns	***
	2	0.172	*	ns	*	ns

H: H test value, ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Table S17. Comparison of CH₄ and NH₃ emissions between seasons in dairy barns during year 1.

Parameter	Dairy farm	Kruskal-Wallis test		Dunn's multiple comparisons test (<i>p</i>)					
		H	<i>p</i>	Winter / Spring	Winter / Summer	Winter / Autumn	Spring / Summer	Spring / Autumn	Summer / Autumn
CH ₄	1	0.500	***	*	*	***	ns	ns	*
	2	0.692	***	ns	ns	ns	*	ns	***
	3	0.602	***	ns	ns	***	*	***	*
NH ₃	1	0.345	***	ns	ns	ns	ns	ns	***
	2	0.723	***	ns	ns	***	ns	ns	**
	3	0.410	***	ns	ns	***	ns	***	ns

H: H test value, ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Table S18. Comparison of CH₄ and NH₃ emissions between seasons in dairy barns during year 2.

Parameter	Dairy farm	Kruskal-Wallis test		Dunn's multiple comparisons test (<i>p</i>)					
		H	<i>p</i>	Winter / Spring	Winter / Summer	Winter / Autumn	Spring / Summer	Spring / Autumn	Summer / Autumn
CH ₄	1	0.344	**	ns	ns	ns	**	**	ns
	2	0.496	***	ns	***	ns	*	ns	***
	3	0.735	***	-	-	-	-	-	***
NH ₃	1	0.329	**	ns	ns	ns	**	*	ns
	2	0.549	***	*	ns	ns	***	ns	***
	3	0.0682	ns	-	-	-	-	-	ns

H: H test value, ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Implications of seasonal and daily variation on methane and ammonia emissions from naturally ventilated dairy cattle barns in a Mediterranean climate: A two-year study

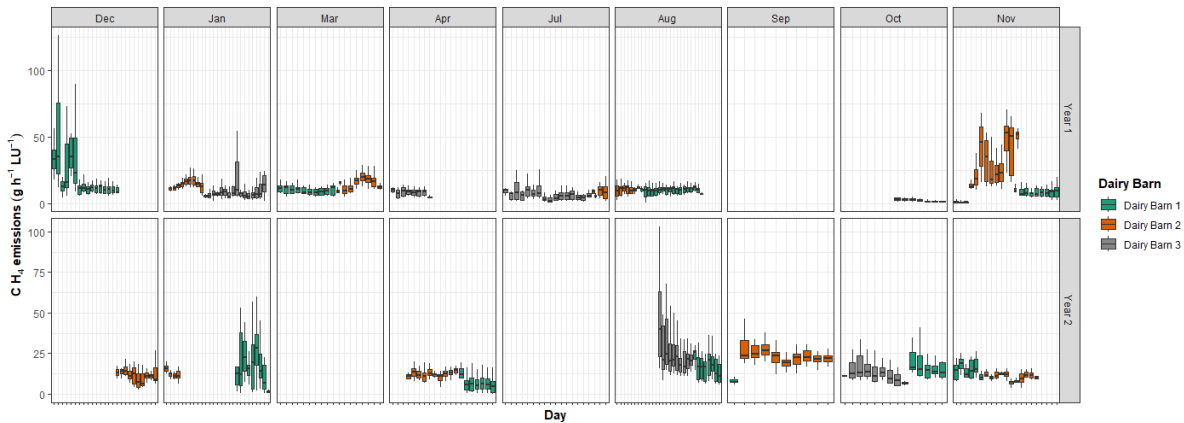


Figure S1 - Hourly CH_4 emissions ($\text{g h}^{-1} \text{LU}^{-1}$) variations in all barns, in year 1 and year 2.

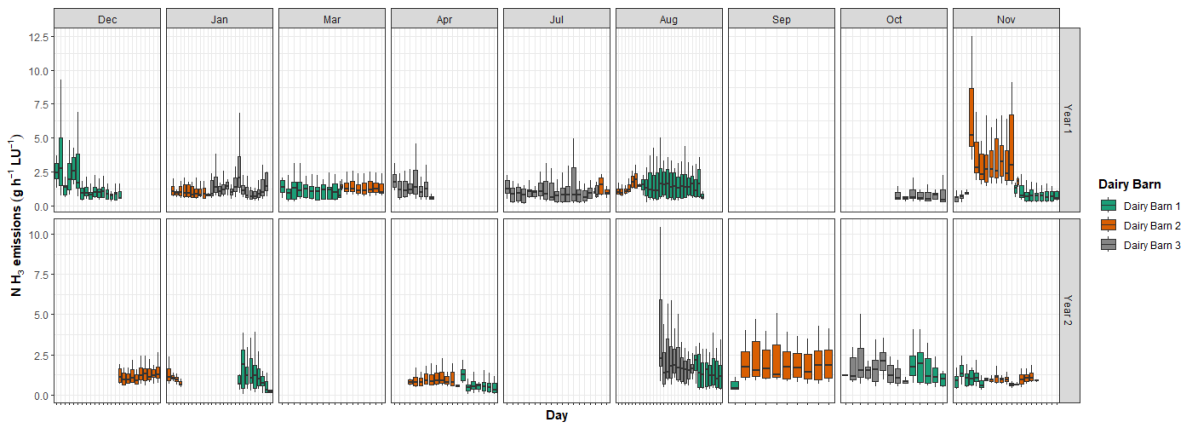


Figure S2 - Hourly NH_3 emissions ($\text{g h}^{-1} \text{LU}^{-1}$) variations in all barns, in year 1 and year 2.

Chapter Six

General Discussion

Context

Climate change is one of the most pressing and complex challenges facing Humanity. Nowadays, taking efforts to improve sustainability is a central piece for all economic sectors. Dairy sector plays an essential role in food security assurance and human nutrition for an ever increasing worldwide population [1, 2]. The awareness of the continuously adapting need to ensure resilience and sustainability makes the sector committed to strategic and effective plans for gaseous emissions reduction, thereby contributing to mitigating climate change.

Considering the main objectives detailed in the introduction, this chapter (Chapter Six) present a holistic discussion of the four previous chapters (Chapter Two, Three, Four and Five).

Explore the effect of biochar as feed additive to reduce CH₄ production from enteric fermentation

The development of feed additives that effectively reduce enteric CH₄ production is currently a hot topic among researchers. The outcomes of Chapter Two reveal the challenge of relating literature findings on the effect of biochar on methanogenesis with specific biochar characteristics or the experimental conditions used in *in vitro* or *in vivo* studies. It is essential to address these gaps in future works to improve the understanding of the biological mechanisms that contribute for the positive effect of biochar on CH₄ reduction.

One example is the effect of biochar pyrolysis temperature on CH₄ reduction. Studies indicate that increasing the pyrolysis temperature enhances surface area of biochar, which can improve biofilm formation and increases the adsorption capacity of microorganisms, nutrients, and gases, ultimately leading to a reduction in CH₄ production. In Chapter Two, we explored 14 *in vitro* short-term incubations studies (up to 48 h), with up to 16% biochar. Some of the studies [3-8] described CH₄ reduction when using biochar produced at very high temperatures (700-1000 °C), while others found no effect [9, 10] or even an increase [11] (Chapter Three) with biochar produced at lower temperatures (350-700 °C). Additionally, Saenab *et al.* [12] observed a decrease in CH₄ production using biochar obtained at 300 °C and Cabeza *et al.* [13] found higher CH₄ production with biochar produced at 700 °C than 550 °C. To highlight that Leng *et al.* [14] was the only study assessing the effect of rumen fluid adapted to biochar. These authors found higher CH₄ reduction with biochar added to unadapted rumen inoculum compared to biochar added to adapted rumen inoculum. Results suggested that biochar enhances the association of microorganism that fermented feed materials more efficiently, and facilitates CH₄ oxidation

by promoting the interaction between methanogenic *archaea* and methanotrophic consortia [15].

Since pyrolysis temperature is the only biochar parameter commonly described, we emphasize the need for a comprehensive biochar characterization to facilitate its enhancement as a feed additive for methanogenesis (Chapter Two). Parameters such as polarity, aromaticity, ash, mineral content, cation exchange capacity, surface charge and textural properties (surface area and pore size) are identified as the key for gaseous removals [16]. In turn, the physicochemical properties of biochar vary according to biomass type and production conditions, thereby enabling its widespread usage [17]. Besides, several activation and modification methods (pre or post pyrolysis) can be used to improve biochar adsorption capacity, including acidification [18, 19].

Various studies have shown differing results regarding the effect of biochar inclusion on ruminal fermentation parameters. In our work [11] (Chapter Three), the inclusion of biochar increased $\text{NH}_3\text{-N}$, *iso*-butyric, *iso*-valeric and valeric acids, decreased total VFA, and kept unaffected total gas production, when compared to control. Previous studies reported varying results concerning total VFA production and individual VFA profiles [13, 20, 21]. These discrepancies may be attributed to the impact of biochar on microbial community and on the fermentation end products profile. In fact, studies on biochar as a soil amendment and its impact on GHG emissions have already indicated that it can provide an habitat for various microorganisms, which may exert selective pressure on microbial community [22, 23]. The effect of long-term biochar supplementation on rumen microbiome was evaluated in few studies. In the work of Teoh *et al.* [21] the abundance of *Methanomethylophilaceae* decreased, suggesting the mitigation potential of CH_4 production with hardwood biochar supplementation. Some authors reported that long-term biochar supplementation did not affect microbial diversity, richness, or relative abundance. However, discriminant analysis indicated specific changes in rumen bacterial families associated with different types of biochar [21, 24, 25]. Few studies have assessed the effects of dietary biochar inclusion on ruminant performance and CH_4 production *in vivo*, similar to *in vitro* studies. The results described in Chapter Two indicate that dietary supplementation with biochar either improve or had no effect on ruminant performance and reduced or did not affect CH_4 production [6, 26-31].

Furthermore, studies demonstrate biochar potential for nutrients recovery from dairy manure [32, 33], and reducing GHG emissions from cattle manure during storage [34]. The potential use of biochar in a cascade value chain make this material extremely versatile [35].

Monitoring GHG and NH₃ emissions from dairy cattle barns

A thorough assessment of gaseous emission sources in cattle production systems is the basis for effective mitigation plans. In Portugal, there's a need for accurate farm-level measurements reflecting management and feeding systems, barns architectonic characteristics and climatic conditions. These not only optimise management practices but also track progress toward national emission reduction plans, as well as monitor the efficacy of existing abatement processes in place and calibrate fairly limits set by international and legally binding agreements [36]. The Chapter Four and Five contribute to improve this understanding on gaseous emissions from naturally ventilated dairy cattle barns in Mediterranean climate.

Assessment of gaseous emissions from dairy cows' excreta under different conditions

Previous studies mainly focus on the effect of urine application rate, temperature, wind speed, relative humidity, and excreta properties on gaseous emissions from dairy cattle' excreta [37-40]. Moreover, strategies for abating gaseous emissions have been evaluated, including floor design, floor cleaning techniques, optimization of dietary protein content, and barn climatization [41]. However, limited information was available on the effects of feeding and milking frequency, as well as stage of lactation, on gaseous emissions, while data on sampling time presented a wide range variation among studies [37-39, 42]. Thus, Chapter Four assessed the effect of the feeding system, lactation stage and sampling time on the emission potential from dairy cows' excreta.

To test the above hypothesis, an experiment was conducted using a laboratory chambers system (LCS) simulating a solid concrete cattle house floor [37]. Individual faeces and urine were collected from two groups of four cows, at peak and post peak lactation, from three commercial farms with distinct feeding systems: total mixed ration (TMR), total mixed ration plus concentrate at robot (TMR + robot), and total mixed ration plus concentrate in automatic feeders (TMR + AF). Faeces and urine samples were collected from each cow at three sampling times, a.m. milking (T8h), at middle day (T12h) and before p. m. milking (T17h). Samples of each stage of lactation, feeding system and sampling time were tested in 9 chambers in three runs of 48-h. Ammonia, N₂O, CO₂ and CH₄ emissions were monitored during 48-h at 20 °C (± 1 °C). TMR+robot led to higher N-N₂O (g/kg ammoniacal-N, ureic N or organic soluble N) than TMR and TMR+AF. It is noteworthy that

the urinary content of TMR+robot had the lowest ureic-N and ammoniacal-N excretions compared to the other two systems. We hypothesized a sum of different pathways to explain these higher N-N₂O emissions from TMR+robot: i) microorganisms may have used NO₃⁻ as final acceptor in the presence of labile C more efficiently; ii) emitting layer may have promoted the N₂O production from N-NH₃ volatilization and from hydrolysis of urea to NH₃; iii) release of N₂O trapped in excreta produced as a product of digestive process at the lower gut.

Ammonia emissions were higher at TMR and TMR+AF systems. This may be explained by the greater urinary ammoniacal-N content observed in these two systems, which is prone to NH₃ volatilization, along with the urinary organic soluble N content, which may have led to mineralization of organic N into NH₃ [43, 44]. With reference to lactation stage, excreta from post peak lactation animals produced higher cumulative emissions of N-N₂O (g/kg total N and ammoniacal-N) and N-NH₃ (g/kg excreta and total N) compared to peak lactation. However, only a tendency to be higher was found for faecal pH from peak lactation cows. Thus, the fluxes profiles were observed to explore these results. Different N-N₂O flux patterns (mg/h/kg total N and ammoniacal-N) in the first hours of deposition was found for both groups of animals. Excreta from peak lactation cows presented a first peak of emission at 2-h after excreta deposition, followed by a second (non-significant) emission peak at 8-h of deposition. In contrast, excreta from post peak lactation only presented a lower N-N₂O emission peak at 2-h deposition. Interestingly, the same emission pattern of peak lactation was found in simulated floors [45], soil [46] and dairy manure [47]. Regarding sampling time, the results indicated that cumulative N-N₂O emissions (g/kg ammoniacal-N) were highest at T17h, whereas faecal ammoniacal-N content tended to be lower, and no differences were detected for urinary ammoniacal-N content. Similar behaviour has been previously described in the literature, which reports that N₂O emissions from barns and barn floors increase in the late afternoon and are correlated with temperature [48, 49].

Peak lactation at T8h presented the highest cumulative C-CH₄ emissions (g/kg excreta, total C, organic C). We did not further investigate individual dry matter intake in this study. However, these results may be attributed to a higher amount of feed ingested by peak lactation animals and/or had a higher ruminal rate of passage during the coolest period of the day, leading to more fermentable OM in the excreta.

Seasonal and daily on-farm CH₄ and NH₃ emissions monitoring in naturally ventilated dairy cattle barns

Enhance the understanding of gaseous emissions from dairy cattle barns will help derive country-specific emission factors (EFs) and adjust appropriate mitigations strategies.

In fact, gaseous emissions from dairy cattle barns differ due to factors such as architectural characteristics, feeding and manure management systems, and climate parameters [50, 51]. Several European countries have been monitoring GHG and NH_3 emissions from naturally ventilated dairy cattle barns, but there is no published data in Portugal.

To address this gap in knowledge, GHG and NH_3 emissions were measured in naturally ventilated dairy barns across three farms with distinct feeding systems (Chapter Five). These measurements were conducted for at least 7 days in each season for two years. In each barn, air samples from 5 indoor locations were drawn by a multipoint sampler (INNOVA 1409) to a photoacoustic infrared multigas monitor (INNOVA 1412). Additionally, indoor temperature, relative humidity and production parameters were also recorded. Daily CH_4 emissions varied among seasons similar to those reported in the work of Tabase *et al.* [36], while Saha *et al.* (2014) found the influence solely of the autumn season [52]. Furthermore, Cortus *et al.* [53] and Huang and Guo [54] identified variations of CH_4 concentrations across seasons, but did not observe the same variations in the emissions. These contrasting results may be explained by architectonic differences among barns, such as the presence or absence of sliding windows and curtains, as well as the density of fans which can affect the stability of indoor parameters and ventilation conditions. Despite including alfalfa in the cow's diet, dairy barn 2 showed no impact on CH_4 emissions. Using this high-quality protein source remains an interesting option to increase economic sustainability with no environmentally adverse effects. Additionally, there were noticeably higher daytime CH_4 emissions, with a persistent peak early in the morning, regardless of feeding times, likely linked to increased animal activity [55].

As for NH_3 , although there were variations between seasons, no strong correlation with exclusively environmental conditions could be found in this study. On the other hand, and similarly to CH_4 , a higher daytime emission was found irrespective of dairy barn or season. An emission peak for NH_3 roughly coinciding with the middle of the day, possibly as a relation to the peak daily temperature, was found in this study, correlating well with a similar emission peak found in the work developed in Chapter Four. We consistently observed this emission peak in both studies (Chapter Four and Five), regardless of the monitoring location (barn floor or overall barn), the season, and the feeding system. The emission factors obtained for CH_4 and NH_3 in this study, 309.5 and 30.6 g/day/livestock unit respectively, are roughly in line with other studies reported in the literature for dairy cattle barns.

Studies involving large volumes of data and under various conditions are extremely important as they provide a deeper understanding of emissions patterns, and correlation between emissions and feeding systems. Additionally, comprehensive data analysis can

enhance decision-making based on data-driven insights, increasing confidence and cost optimization leading to enhanced profitability and economic sustainability.

Conclusions

This PhD thesis was motivated by the need to balance the increasing global demand for high-quality animal protein with the need to improve dairy cattle sustainability, specifically by reducing GHG and NH_3 emissions. In this study, the inclusion of biochar as a feed additive was analysed, using a combined approach of literature review (Chapter Two) and experimental *in vitro* study with two biochar sources at two inclusion levels (Chapter Three). Results from *in vitro* study showed that the conditions tested (two types of biochar at two inclusion levels) led to increasing CH_4 production when compared to the control. It can be concluded that, although the potential benefit of biochar is evident in the literature, the lack of standardization and deeper knowledge, particularly on the mechanisms under which biochar can produce a methane reduction, is currently scarce, greatly limiting potential applications. The present thesis also explored gaseous emissions from dairy cows' excreta, in a simulated barn floor (Chapter Four). Based on the results, it can be concluded that the feeding system, stage of lactation and sampling time greatly affect $\text{N-N}_2\text{O}$ and N-NH_3 emissions. These results highlight the importance of considering these parameters in practical strategy plans to reduce gaseous emissions at a farm level. Measurements of CH_4 and NH_3 emissions were undertaken on farm to explore seasonal and daily variations in naturally ventilated dairy cattle barns. Additionally, emission factors for CH_4 and NH_3 were also assessed. It can be concluded that CH_4 and NH_3 emissions were higher during daytime with high intra-hour variability. Besides, the study also made it possible to provide up-to-date EF for CH_4 and NH_3 . Emission factor obtained for CH_4 was 309.5, which is in line with other studies, resulting in EF from 259.2 g/day in Central Europe to 300 g/day in Canada. Regarding NH_3 , the EF obtained was 30.6 g/day, within the range described in the literature, with studies varying between 19 g/day in Poland and 80 g/day in Denmark.

This was the first study assessing CH_4 and the second assessing NH_3 emissions from naturally ventilated barns performed in Portugal. It contributed to the understanding of similarities and differences among barns in seasonal and daily gaseous emissions. This information is essential for developing detailed, robust, and effective mitigation plans. Overall, this PhD thesis contributed to the innovation and progress of the dairy sector by identifying the past scientific advances and challenges, and establishing future research opportunities, particularly towards a more sustainable production.

Future Perspectives

Throughout this thesis, several promising avenues for future research in the dairy cattle sector were identified. This section highlights the most important of these avenues and their potential effects, divided by main topics and presented in concise bullet points.

On biochar inclusion as feed additive for methanogenesis:

- Exploring key and measurable characteristics of biochar production process and biochar itself, to ensure a complete characterization enhancing the experimental designs standardization within this field of research and improving results comparability;
- Focus on expanding the knowledge of the effect of biomass type and production conditions on biochar adsorption capability, to develop understanding on mechanism by which biochar reduce CH₄ production;
- Explore the potential cascade effect of biochar, such as effect of dietary biochar inclusion not only on enteric CH₄ production, but also on gaseous emission potential from manure, or in milk production;
- Studies to investigate in-depth the effect of biochar supplementation on microbial communities are also necessary, both in rumen and faeces.

Greenhouse gases and ammonia emission monitoring:

- Studies on the effect of the most promising feed additives to reduce gaseous emissions from the barn floor to accelerate emission targets for dairy cattle sector;
- Long-term studies to monitor GHG and NH₃ emissions allowing the assessment of EFs as well as seasonal and daily emissions, to strengthen the results obtained with this first study performed in Portugal;
- The inclusion of other specific parameters in the long-term studies would allow to deepen specific aspects, such as higher number of temperature and relative humidity sensors to improve the barn sampling to better represent the whole area, or consider specific chemical analysis on the animals' diet to estimate EF from enteric CH₄ from dairy cattle.

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