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DIETARY STRATEGIES TO INCREASE ENVIRONMENTAL SUSTAINABILITY OF DAIRY COW MILK PRODUCTION

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ABSTRACT

The livestock sector is facing different challenges, and the demand for higher sustainability seems to be one of the most urgent. This PhD project debated, in particular, the environmental impacts related to ruminant nutrition, focusing on dairy cows, since nutrition is bound tightly to two of the most important sources of impact: enteric CH_4 emission and land use change (LUC). Enteric CH_4 emission from ruminants represents 29-38% of the total (anthropic + natural) emission of this powerful (21 CO_2 equivalent) greenhouse gas. The production of CH_4 is a physiological process used by ruminants to discharge the [H] resulting from rumen fermentation. Different strategies can be implemented to mitigate this impact, and they can be roughly grouped into three main categories: animal and feed management, diet formulation, and rumen manipulation. The second issue investigated in the project is the high reliance of European livestock on soybean meal as a protein source for diet formulation. A total of 30 million tonnes of this feedstuff was imported into Europe in 2020. The main countries of origin are in South America (65% of total import), where 20% of soybean meal production was linked with deforestation (and consequently LUC) in the last decades. Clearing these areas means loss of carbon sink and emission of CO_2 in the atmosphere. Other feedstuffs, like grain legumes, oilseed meals alternative to soybean, and high quality forages could be considered to provide protein feed with a lower environmental cost.

In this context, the PhD project was developed as follows:

- To address the problem of CH_4 emission, plant essential oils, as modulators of rumen fermentation, were evaluated (Experiment 1). Furthermore, the effect on CH_4 emission of different forages in the diet of dairy cows was investigated (Experiment 2). For validation of mitigation strategies and inventory computation of emissions at a national scale, country-specific equations to quantify CH_4 emission were evaluated (Experiment 3).
- To address the problem of soybean meal environmental impact, soybean silage and responsible soybean meal (not connected with land use change) were evaluated as protein source alternatives to soybean meal in the diet of lactating cows (Experiments 4 and 5).

Enteric methane direct emission

In the first experiment, *Achille moschata* essential oil and its main pure components, namely bornyl acetate, camphor, and eucalyptol, were evaluated in an in vitro experiment. The trial comprehended a short-term *in vitro* incubation (48 h), with 200 mg of compound per L of inoculum, and a long-term one by continuous fermenter (9 d), with 100 mg/L for each compound. In the first incubation, no differences due to the treatments were found for in vitro gas production (on average, 30.4 mL/200 mg DM, $P = 0.772$ at 24 h and 45.2 mL/200 mg DM, $P = 0.545$ at 48 h). Camphor and eucalyptol reduced CH_4 production when expressed as % of gas production at 48 h ($P < 0.05$): -7.4% and -7% compared to control. In the second incubation, CH_4 was reduced by eucalyptol (-18%, $P < 0.05$). Regarding volatile fatty acids, the main effects were a decrease of total production for camphor (-19.5%, $P < 0.05$) and an increase in acetate production at 9 d with bornyl acetate and camphor (+13% and 7.6%, respectively, $P < 0.05$) compared to control. Total protozoa count was increased compared to the control (on average: +37%, $P = 0.006$, at 48 h and +48%, $P < 0.001$, at 9 d) with all the pure compounds tested. In the short-term incubation, all the treatments reduced Bacteroidetes (30.3%, on average, vs. 37.1% of control, $P = 0.014$) and Firmicutes (26.3%, on average, vs. 30.7% of control, $P = 0.031$) abundances but increased Proteobacteria (36.0%, on average, vs. 22.5% of control, $P = 0.014$). In the long-term incubation, eucalyptol increased the genus *Ruminococcus* abundance (2.60% vs. 1.18% of control, $P = 0.011$). An adaptation at long time incubation was observed. In particular, considering eucalyptol addition at 9 d incubation, VFA production was reduced (26.8 vs. 33.3 mmol of control, $P < 0.05$) contrary to the 48 h incubation ($P = 0.189$). Furthermore, the treatments affected protozoa genera relative abundances at 24 h (increased abundance for *Entodinium* with all the treatments, $P < 0.001$, and reduced for *Diplodinium,* $P = 0.001$); at 9 d, instead, protozoa genera relative abundances were not affected by the treatment. The additives tested showed potential in reducing CH_4 production without compromising the overall fermentation efficiency.

A meta-analysis (Experiment 2) investigated the effects on lactation performance and enteric CH_4 of the main forage included in the diet. In the dataset, composed of in vivo experiments, four main forage bases were evaluated: corn silage, alfalfa silage, grass silage, and green forage. Cows fed corn, and alfalfa silages had the highest DMI (21.9 and 22.0 kg/d, $P < 0.05$) and milk yield (29.7 and 30.4 kg/d, $P < 0.05$). On the opposite, NDF digestibility was highest for grass silage and green forage (67.6% and 73.1%, $P < 0.05$) than corn and alfalfa silages (51.8% on average). CH_4 production was lower ($P < 0.05$) for green forage (332 g/d) than the silage diets (on average 438 g/d). Instead, corn silage and alfalfa silage gave the lowest CH_4 per kg of milk yield (14.2 g/kg and 14.9 g/kg, $P < 0.05$). Considering CH_4 per kg of DMI, the only difference was between corn silage and grass silage (19.7 g/kg vs. 21.3 g/kg respectively for corn and grass silage, $P < 0.05$). Finally, prediction models for CH_4 production were obtained through a step-wise multi regression. In particular, the models for the prediction of:

CH_4 in g/d (CH_4 = - 65.3(±63.7) + 11.6(±1.67) × DMI - 4.47(±1.09) × CP - 0.86(±0.33) × Starch + 2.62(±0.78) × OM digestibility + 30.8(±9.45) × Milk fat)

and for

CH_4 in g/kg of milk yield (CH_4/milk yield = - 55.5(±20.1) - 0.37(±0.13) × DMI + 0.18(±0.05) × Total forage inclusion on diet DM - 0.10(±0.04) × Inclusion of the main forage on diet DM + 0.48(±0.21) × OM + 0.14(±0.06) × NDF + 1.98(±0.86) × Milk fat +4.34(±1.66) × Milk protein)

showed high precision (R^2 = 95.4% and 88.6%, respectively), but the best AIC value (320) was found for the model predicting CH_4 in g/kg DMI:

CH_4/kg DMI = 6.16(±3.89) - 0.36(±0.03) × CP + 0.12(±0.05) ×OM digestibility + 3.77(±0.56) × Milk fat - 3.94(±1.07) × Milk fat yield.

A dataset (66 observations in total) of three in vivo experiments conducted in Italy on lactating cows in respiration chambers was built to evaluate IPCC Tier 2 equations to estimate enteric CH_4 production (Experiment 3). In the dataset, the CH_4 conversion factor (conversion of gross energy intake into enteric CH_4 energy) was lowest for a diet based on grass and alfalfa silages (5.05%, $P < 0.05$), while the others values ranged between 5.41 and 5.92%. On average, energy digestibility was 69.0% across the dataset, but the diet based on hays had a lower value (64.8%, $P < 0.05$). The IPCC (2019) Tier 2 (conversion factor = 5.7% or 6.1% for diet with NDF concentration < 35% or >35%, respectively; digestible energy = 70%) gave, on average, a value of CH_4 production not statistically different from the ones measured in vivo (382 vs. 388 g/d in vivo, $P > 0.05$). The IPCC (2006) Tier 2 (conversion factor = 6.5%, digestible energy = 70%) over-predicted CH_4 emission (428 vs. 388 g/d in vivo, $P < 0.05$; $\mu = -1.05$). The most precise models were the two considering digestible energy equal to 70% and average values of conversion factor for IPCC (2006) and IPCC (2019) (R = 0.630); the most accurate models was the one considering a conversion factor equal to 5.7% and energy digestibility measured in vivo (Cb = 0.995). Overall, the best performance among the predicting models tested was for the one based on a conversion factor equal to 5.7% and energy digestibility of 70% (CCC = 0.579 and RMPSE = 9.10%).

Use of alternative protein source to conventional soybean meal

The dietary inclusion of soybean silage in partial replacement of soybean meal for dairy cows was evaluated in vivo in lactating cow diets (Experiment 4). Cows were fed two diets, one with 12.4% of DM from soybean silage in substitution of 35% of the soybean meal of the control diet. The treatment did not affect DMI and milk yield (on average, 23.7 kg/d, $P = 0.659$, and 33.0 kg/d, $P = 0.377$, respectively). Cows fed the soybean silage diet had lower milk protein concentration (3.43% vs. 3.55% of the control, $P < 0.001$) and higher milk urea (30.5 vs. 28.7 mg/dL, $P = 0.002$). The soybean silage had lower nutrient digestibility than the control: DMD 65.2% vs. 68.6%, OMD 66.4% vs. 69.8%, NDFD 31.5% vs. 38.8% (respectively for soybean silage and control diet; $P < 0.001$ for all of them). Regarding N balance, cows fed soybean silage excreted more nitrogen in the urines (32.3 % of N intake vs. 28.9%, $P = 0.005$) and less in the milk (31.3% vs. 32.7%, $P = 0.003$) than the control. When used as a protein source alternative to soybean meal, soybean silage sustained comparable milk production, but NDF digestibility and N use efficiency should be improved.

The environmental impact of the use of soybean silage in comparison to a control diet with soybean meal as the main protein source was evaluated through an LCA approach (Experiment 5). In addition, two scenarios were included in the study, considering the two diets mentioned before, but with soybean meal not connected to LUC (responsible soybean meal). Regarding the single forages, soybean silage had higher global warming potential than alfalfa hay (477 vs. 201 kg CO_2eq/ton DM), also when this was expressed per tonnes of protein production (2439 and 1034 kg CO_2eq/ton CP, respectively), probably due to the lower contribution of the cultivation phase for alfalfa, being a multi-year crop. The scenario with soybean silage reduced the global warming potential per kg of fat and protein corrected milk (1.17 kg CO_2eq) compared to the control (1.38 kg CO_2eq). Responsible soybean meal reduced the global warming potential per kg of fat and protein corrected milk (1.13 kg CO_2eq/kg vs. 1.38 of the scenario with the control diet). Overall, the best result per kg of fat and protein corrected milk was obtained when responsible soybean meal and soybean silage were used in combination (1.01 kg CO_2eq). Also, when global warming potential was evaluated per daily fed TMR, the impact was lowest for the scenario with responsible soybean meal (13.4 kg CO_2eq/d) due to the lower contribution of soybean meal to the total impact (11% vs. 43% of the control). Therefore, the two alternative protein sources tested should be preferred when considering environmental impact compared to conventional soybean meals.

1. Introduction

1.1. Agriculture challenges

The agriculture sector has to deal with several challenges, which will be even more urgent in the future. In particular, the World Resources Institute (report of Searchinger et al. 2019) identified the closing of three gaps as primary goals for this sector:

- The food gap, with an increase of the demand of crop calories equal to + 56% from 2010 to 2050;
- The land gap, with an increase of 593 million hectares required for crop and pasture from 2010 to 2050;
- The greenhouse gas (GHG) emissions gap, calculated as the difference between the GHG emitted by agriculture and the threshold of emission required to keep global warming below 2°C above pre-industrial temperatures. In 2050 this gap is estimated to be 11 gigatons of carbon dioxide equivalent (Gt CO2e).

Therefore, the first challenge for agriculture is to feed a growing population, which is expected to grow by more than 9 billion by 2050 (FAO 2009). The importance of this mission was underlined by the United Nations document (UN, General Assembly, 2015) defining the Sustainable Developments Goals (SDGs) to be reached by 2030. Though addressing a multiplicity of themes (e.g. social, economic, and environmental), some of these goals seem to first-hand involve the agricultural sector. The second SDG deals with the theme of food security, the need to end hunger, and improving the nutrition of the more delicate categories (children, adolescents, pregnant and lactating women). This is an urgent problem because, in 2018, the prevalence of undernourishment was still 8.9% and thus, despite marked improvements in the last 20 years, over 675 million people were still suffering from that (World Bank). Therefore, agriculture was asked to double its productivity by 2030 (UN, General Assembly, 2015).

However, besides food security, food sovereignty has to be considered as well. With the income growth in some developing countries, the alimentary choices are changing, with the demand for more diversified diets. This tendency will increase demand for vegetables, fruits, meat (from 41 kg per capita in 2009 to 52 kg in 2050), dairy, and fish (FAO, 2009). As a result of these two trends, more people to feed and more demand for less "efficient" food (as the conversion efficiency of the plant into the animal matter is only about 10% according to Godfray et al., 2010), agriculture will have to compete even more for resources and will be forced to increase its productivity. In this scenario, agricultural research plays a strategic role in spreading knowledge and innovations to farmers and other producers to increase yield and productivity.

The second great challenge is to use efficiently and responsibly the resources available. According to the FAO report "How to Feed the World in 2050" of 2009, despite the higher demand for food, and thus of land and water, agriculture will have to compete for these two resources with the expanding of urban areas and with arising needs like preserving natural habitats and maintaining biodiversity. The arable land per person halved from 1960' until now (from 0.361 ha per person of 1961 to 0.184 of 2018; World Bank), while renewable internal freshwater per capita was reduced even more (from about 13,400 cubic meters of 1962 to about 5,700 of 2017; World Bank). Globally, agriculture is the largest consumer of water, given that the withdrawals from this sector represent 71.3% of total withdrawals (World Bank), and the competition is expected to increase due to water scarcity and depletion. Due to the competition for resources, it is evident that the approach should maximize productivity, considering the environmental outcomes (Godfray et al. 2010).

Indeed, the environmental impact of anthropic activities has to be reduced, and this represents the third goal of the sector. The AFOLU (Agriculture, Forestry and Other Land Use) sector is responsible for about one-quarter of anthropogenic GHG, with the main causes represented by deforestation, livestock, soil, and nutrient management (Smith et al. 2014). Consumers are more aware and sensitive to this theme, mainly in the developed countries and especially concerning the environmental impact of animal food production; that is the reason for the rise of diets like vegetarianism and the emergence of synthetic milk and meat, which represent a further concern for the ruminant livestock industry, in particular (Beauchemin et al. 2020).

Like every human activity, livestock farming has a role in environmental impact and climate change. Industrialized agriculture, and so by extension livestock farming, was accused, together with fossil fuels reliance, to push human activities outside the safe operating space, where average temperatures, freshwater availability, and biogeochemical flows equilibrium is no longer guaranteed (Rockström et al. 2009).

The environmental pollution linked to agriculture, and livestock in particular, happens on different levels.

Air pollution is due to the emissions of gases like carbon monoxide, chlorofluorocarbons, ammonia, nitrogen oxides, sulphur dioxide, and volatile organic compounds, some of them returning to the earth in the form of acid rain and snow. Livestock contributes to the net release of carbon into the atmosphere, mainly through the burning of fossil fuels and land-use change (LUC). This sector is also impactful for enteric CH_4 emission (see below), but this is considered part of animal respiration, which, by convention, is not counted as a net source of carbon: the carbon exhaled by the animal returns to it in the form of vegetable biomass (Rojas-Downing et al. 2017). That is why indirect sources of carbon are more critical than direct ones and they include mainly feed productions (fossil fuels to produce fertilizers; fossil fuel to produce feed; LUC for feed production and grazing) and making and delivering the end product (fossil fuel to produce and transport processed and refrigerated animal products).

Considering the GHG emission, CO_2 from the livestock sector accounts for 9% of global anthropogenic emissions, methane is 35-40% of total emissions, and nitrous oxide is about 65%. The main sources of GHG from the livestock sector are presented in Figure 1 (Steinfeld et al. 2006). According to their lifetime in the atmosphere and their global warming potential (GWP), these gases have a different contribution to the greenhouse effect: CH_4 has a lifetime in the atmosphere of 12 years and a GWP of 21 CO_2e in a 100 years horizon, while N_2O has a lifetime of 120 years and a GWP of 310 CO_2e (United Nation – Climate Change). Carbon dioxide has a variable lifetime in the atmosphere, ranging from centuries to millennia (Reisinger et al. 2017).

Despite the shorter lifetime of CH_4 and N_2O, non-CO_2 GHG emissions are estimated to increase and to become the most significant share of GHG emissions due to the expected implementation of strategies for CO_2 mitigation. In particular, in the study of Gernaat et al. (2015), different scenarios were evaluated in order to understand the possible incidence of non-CO_2 emissions and the potential to mitigate them. In the scenario where the highest mitigation of CO_2 emissions was considered, total emissions become net negative by 2100. However, for this reason, the relative contribution of non-CO_2 gases was expected to increase above 100%. The authors showed that the main sectors causing the increase of non-CO_2 emissions were energy supply and livestock. According to this study, the agricultural sector had lower mitigation potential, estimated to be between 9 and 43% across the different scenarios. This trend was also confirmed in the study of Reisinger and Clark (2017). The authors considered only direct emissions from livestock and determined the fraction of non-CO_2 emissions within total emissions to date and in the future under different scenarios. From 19% of global warming potential of 2010 (considered as starting point for the study), non-CO_2 livestock emissions could either be reduced to 5% in 2100, in case the emissions from other sectors increase unabated, or remain the 18% in case the other sectors would reach carbon neutrality as expected to keep global warming below 2°C. The authors hypothesized that considering indirect emissions as well, the role of livestock in global warming potential could be even more important.

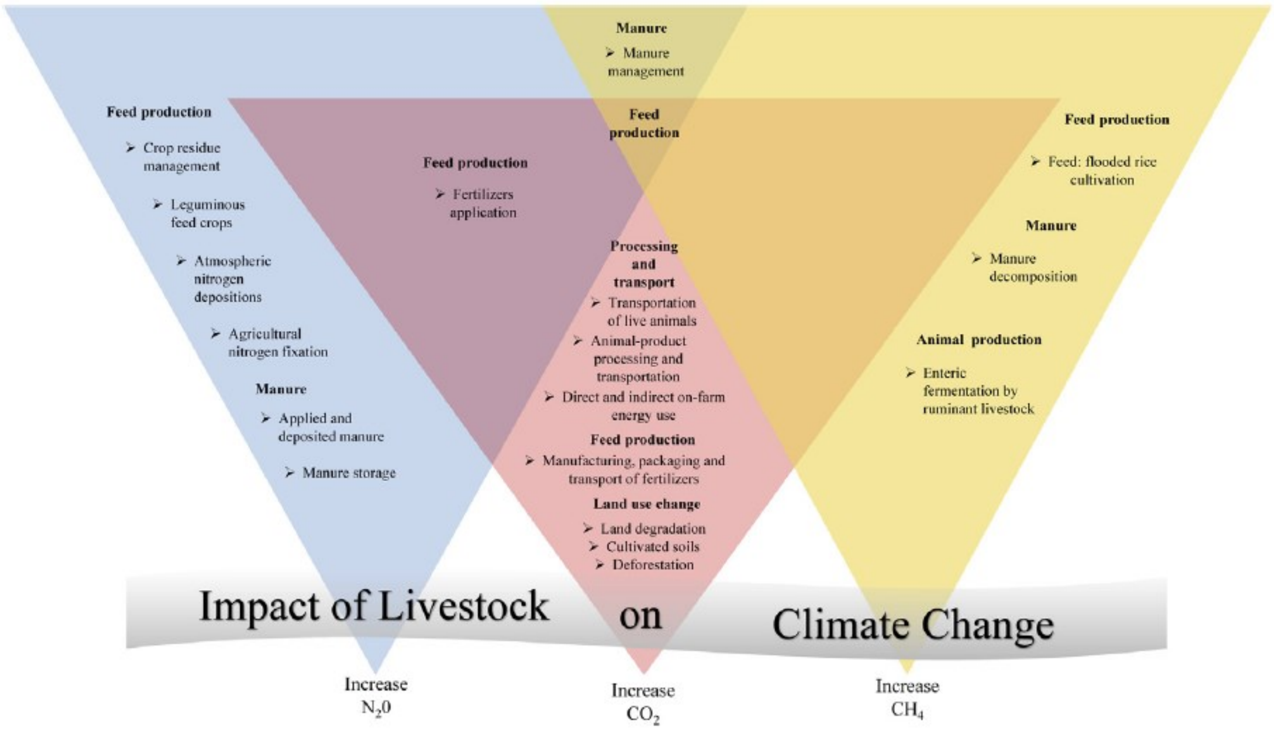


Figure 1: main sources of GHG from livestock. Source: Rojas-Downing et al. 2017.

1.2. Enteric methane emission from dairy cows

1.2.1. Methanogenesis

According to the data elaborated by Jackson et al. (2020), based on the analysis of Saunois et al. (2020) for the year 2017, CH_4 of anthropic origin represents 51 to 61% (according to the estimation method) of total CH_4 emission. Among anthropic sources, agriculture was the most important one, contributing 29 to 38% of total emissions (natural + anthropic), and among agricultural sources, enteric and manure fermentation were the main cause (15% of total emission) with 115 million tons per year. The EU elaborated a document to address CH_4 emissions (EU methane strategy; European Commission), stressing the importance of taking action against this GHG to reach the climate neutrality goal by 2050. This is one of the goals of the Paris Agreement, the international treaty signed in 2015 by 196 Parties to keep global warming below 2°C compared to the pre-industrial level (United Nations – Climate Change).

Methane production represents the main sink of [H] in the rumen, and methanogenesis reduces CO_2 and other one-carbon compounds via the hydrogenotrophic pathway to CH_4. This metabolic process is required because dietary carbohydrates like cellulose, hemicellulose, and starch undergo a catabolic process in the rumen which hydrolyses them to glucose, other hexoses, and pentoses, leading to volatile fatty acids (VFA) and CO_2 as final fermentation products. Along this process, metabolic hydrogen ([H]) is released. Excessive accumulation of [H] might inhibit rumen fermentation by the impairment of microbial enzymes, like NADH dehydrogenase, that are involved in electron transfer, leading to an accumulation of reduced forms of these enzymes (Morgavi et al. 2010). Oxidation is run by hydrogenase activity and formation of H_2, molecular hydrogen, which could be either dissolved and thus available for rumen microorganisms, or in gaseous form. Methanogen archaea cause a rapid turnover of H_2, using this atom to reduce CO_2 and other one-carbon compounds. The hydrogenotrophic pathway is the metabolic way used by most rumen methanogens, with electrons from H_2 that reduce CO_2 to CH_4. Many hydrogenotrophic methanogens can also use formate as an electron donor, with formate that has to be oxidized to CO_2 prior to CH_4 formation. Besides this main methanogenic pathway, other two, even if less important, which lead to enteric CH_4 production are the methylotrophic and the acetoclastic pathways, as reviewed by Huws et al. (2018). In the methylotrophic pathway, methanogens use a methyl-group containing compound (e.g. methanol, methylated amines, and methylated sulphides) as substrates. These compounds enter into the methanogenesis pathway and are finally reduced to CH_4. In the third pathway, the substrate used is acetate, which is broken down into carboxyl and methyl groups. The methyl group is then reduced to CH_4. (Liu and Whitman 2008) As an example, *Methanosarcina barkeri* CM1 methanogenic pathways are reported (Figure 2) because *Methanosarcina* species are versatile methanogenic archaea and they can follow each of the three pathway mentioned above to obtain energy via CH_4 production (Lambie et al. 2015).

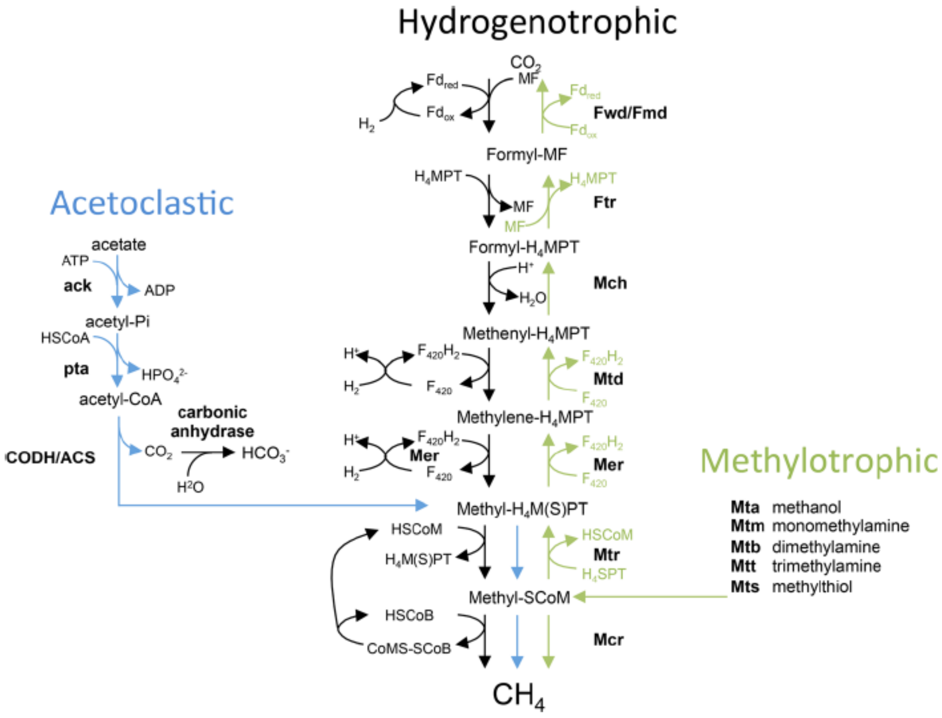


Figure 2: acetoclastic, hydrogenotrophic, and methylotrophic pathways of *Methanosarcina barkeri* CM1 leading to CH_4 in the rumen. Source: Lambie et al. 2015.

As explained above, this necessary process leads to the production of a harmful gas (CH_4) for the environment, but it is also an inefficiency for the cow. According to the Intergovernmental Panel on Climate Change (IPCC 2019), CH_4 is a loss of the energy ingested by the animal, ranging from 5.7 to 6.5% of gross energy intake (GEI), even if the actual improvement in energy efficiency, with reduced enteric CH_4 emission, is still debated. For example, in their review, Beauchemin et al. (2020) estimated the metabolizable energy (ME) saved by the cow when enteric emission is abated. They hypothesized an initial loss as CH_4 between 3 and 17% of total GEI and the emission mitigation, which can be considered moderate (25% abatement). The result of this calculation is that ME could be improved by 0.75 to 4.25%. However, ME is converted into net energy of production with efficiency usually lower than 65%. For this reason, the actual improvement of net energy available for the animal, deriving from CH_4 abatement, would be at most the 65% of 4.25%. Thus, a consistent effect on energy saving for the cow could be appreciated if the reduction of methanogenesis would be more extreme, as Ungerfeld (2018) stated. This aspect may represent another critical point because producers would be more encouraged to apply mitigation strategies if this means having economic benefits in terms of productivity gains. However, no relationship between inhibiting methanogenesis and DMI-adjusted ECM (energy corrected milk) production was detected in the meta-analysis of Ungerfeld (2018). Thus, the best way to encourage the application of mitigation strategies would be a tax on carbon emission or certification for products derived from reduced emissions practices, betting on the will of consumers to pay more for more sustainable food (Peyraud and MacLeod 2020).

1.2.2. Dietary factors

Variation of the amount of CH_4 emitted can be due to several factors. However, dietary aspects, including nutrition, feed management and diet formulation, can highly affect the production of this GHG. The main factor is DMI, as the feeding level can explain a large portion of the variability in CH_4 production, from 52 to 64% when cows are fed ad libitum, according to Knapp et al. (2014). To sustain the concept that DMI is highly correlated with CH_4 production, the high similarity in the average value of CH_4 yield across different datasets can be considered. Hirstov et al. (2018) found an average of 20.1 g/kg DMI in a massive dataset of 4152 observations (Figure 3).

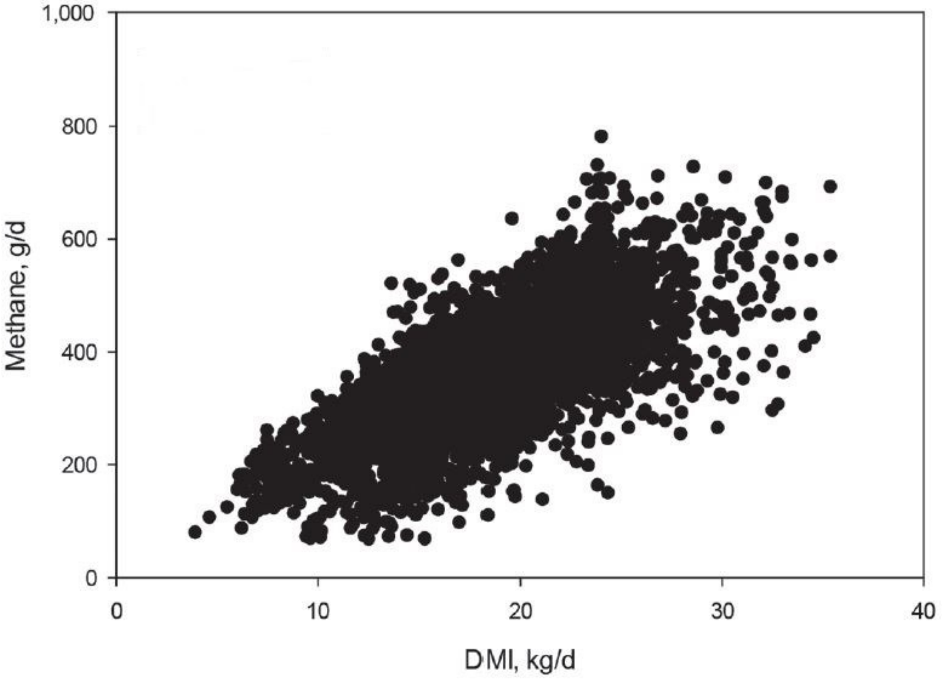


Figure 3: relationship between methane production (g/d) and DMI (kg/d) in the Global Network Database (Niu et al. 2018). Source: Hristov et al 2018.

Charmley et al. (2016) found a value of 20.7 g/kg DMI in a dataset comprising 1033 observations, 21.0 g/kg DMI considering dairy cows only. In Hristov et al. (2013), this value was equal to 19.1 g/kg DMI, and the observations in the dataset were 377. In particular, in the last two studies mentioned, the relationship between DMI and CH_4 production was found to be linear.

The type of carbohydrates included in the diet is another factor that can affect methane emission, with more fermentable carbohydrates associated with higher DMI and milk yield but lower CH_4 yields. The review of Knapp et al. (2014) summarised how cellulose, hemicellulose, and lignin, and thus high-forage diets, favor CH_4 production; the opposite for starch and thus high-concentrate diets. These concepts are exemplified in the paper of Zhao et al. (2012): the authors tested increasing inclusions of beet pulp, a source of NDSF (neutral detergent-soluble fiber), in substitution of ground corn and wheat bran, sources of starch and NDF, respectively, in diets used as substrates for in vitro rumen fermentation. The four diets tested had nearly the same concentration of NDF (33.3%) and NFC (41.2% on average) but a different proportion of starch and NDSF. It was found that the diet with lower NDSF concentration resulted in the lowest amount of CH_4 per mol of VFA, with propionate production following the opposite trend.

Furthermore, high-digestible fibre is more methanogen than low-digestible fibre (Guyader et al. 2014) and tropical grasses (C4) are more prone to CH_4 production than temperate forages (C3) (Archiméde et al. 2011).

Feed particle size is another predisposing factor because smaller particles (the result of feed processing, for example) can bypass the rumen without being digested (Russel and Hespell, 1981) and thus reduce the amount of total rumen fermentation, including methanogenesis. Regarding these factors, De Boever et al. (2017) tested two different corn silage in the diet of dairy cows, one of them having lower NDF digestibility (NDFD) (51.1% vs. 55.1%) and higher starch concentration (38.2% vs. 36.3%). As a result, cows fed the diet including this silage produced less CH_4 (336 g/d vs. 376 g/d), as a result of lower NDFD in the rumen and higher escape of starch from rumen fermentation.

1.2.3. Methods to measure enteric CH_4

The accurate measurement or estimation of CH_4 emission is important for several purposes, including evaluating mitigation strategies and decision making. Hill et al. (2015) reviewed that CH_4 emission can be measured through in vivo, in situ, and in vitro methods or can be estimated through prediction models.

The most used in vivo technique is the direct measurement through respiration chambers (Figure 4b) and head or face masks. Respiration chambers are the gold standard used to develop predicting models and equations because they allow complete measurement of the gaseous exchange and emissions from the animal. However, this method is expensive, it permits only short time measurement periods (e.g. 3 days) for a single animal, and the chambers represent a controlled but artificial environment, which might affect the behavior of the cow, not reflecting the free-range pattern (Storm et al. 2012). Instead, a modified head mask method can be used for grazing animals. It allows a spot sampling measurement, where a larger number of experimental animals can overcome the high variability of these short-term measurements. One example of this system is GreenFeed (C-Lock Inc., Rapid City, SD, USA) (Figure 4a), which takes breath samples when the cows visit a bait station: it appeared evident that the animals have to be trained in order to use the GreenFeed system, and this may be a limitation.

Gas measurements in situ can be done through the sulphur hexafluoride (SF_6) techniques (Figure 4c). Small permeation tubes filled with this gas are placed inside the rumen while the sampling apparatus consists of a collection canister, a halter and capillary tubing. This technique estimates CH_4 emission starting from a known emission rate of SF_6, which is used as a tracer gas. The equipment needed can be rather expensive, and only a small number of animals can be evaluated for a period of 5-7 days.

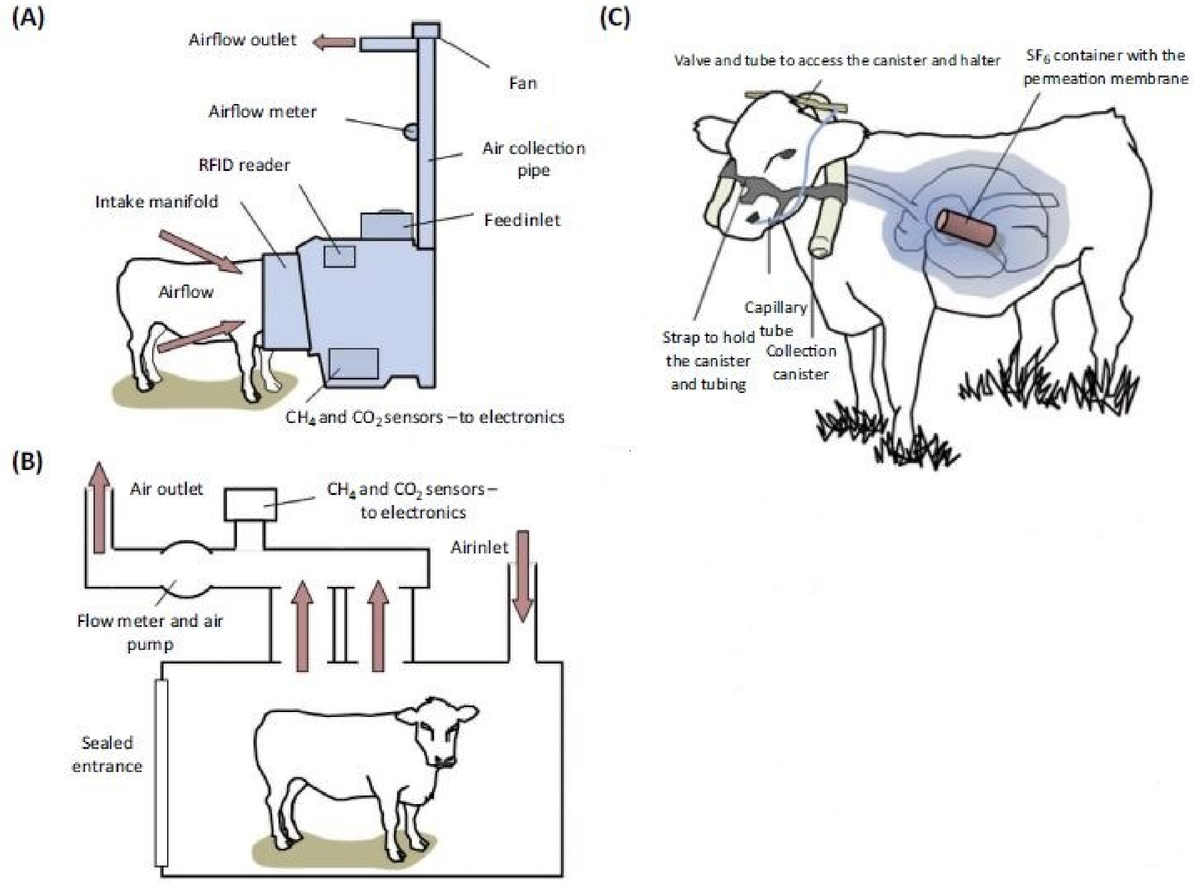


Figure 4: methods to measure CH_4 production in vivo: a) Greenfeed; b) Respiration chambers; c) SF_6 technique. Source: Hill et al. 2015.

The in vitro gas production technique is another option for measuring enteric CH_4 emission. This method simulates ruminal fermentation of feedstuff under controlled laboratory conditions. It typically requires fresh rumen fluid in order to employ natural rumen microbes in the fermentation batch systems. The rumen fluid can be obtained from cannulated cows, esophageal tubing on intact animals, or slaughtered animals. Through this method, many substrates or treatments can be incubated rapidly and inexpensively, but other important factors, like the level of intake of the animals and the physicochemical nature of the feed, are not taken into account compared to in vivo methods.

Methane emission can also be estimated through proxy or prediction models, with the advantage of not requiring invasive operation for the animal or large experimental set-ups (Storm et al. 2012). The proxy methods aim to find parameters highly correlated with CH_4 emission but easily obtainable and measurable in biological samples like milk or faeces. Prediction models might be more complex and consider multiple variables related to animal characteristics, ration chemical composition, digestibility and ingredients, intake level, or others (Storm et al. 2012). Two weak points of these methods are the risk of oversimplification of the complex process of enteric methanogenesis and that direct comparison with other approaches (e.g. respiration chambers) has to be performed.

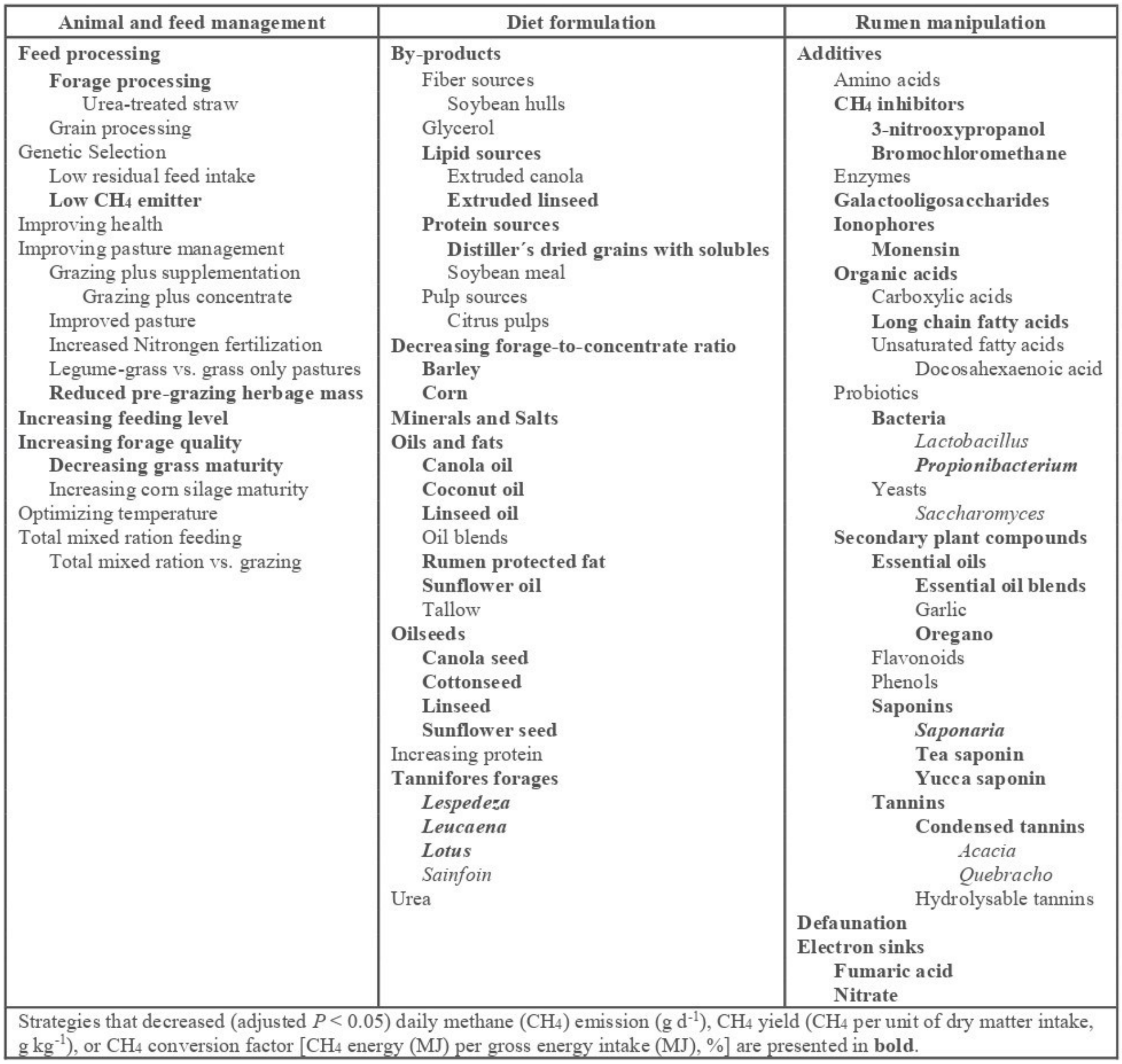
Among the most used models, there are the ones elaborated by the IPCC. The IPCC is an independent international scientific body established by the World Meteorological Organization and the United Nations

Environment Programme (UNEP). Climate change is one of the most important global environmental challenges because it produces irreversible and long-term consequences for both environment and humanity. For this reason, it concerns many stakeholders, including policy makers, industry, non-governmental organizations, citizens, and mass media. Furthermore, climate change is an interdisciplinary research field and thus requires collaborations within the global scientific community. The purpose of the constitution of IPCC was to find a global strategy to deal with this challenge and deepen the understanding of the risk of human-induced climate change (Ravindranath 2010). The aim of IPCC is to provide internationally agreed methodologies to estimate GHG emissions, including enteric CH_4. The IPCC has three levels of complexity/accuracy for the predicting equations (Tiers 1, 2 and 3), which can be applied according to the data available. The simpler approach, Tier 1, involves using a fixed emission factor (i.e. g of CH_4/(head x year)) typical of a livestock category and of a geographic region, to be multiplied by the number of heads belonging to that category. Tier 2 required data more detailed and difficult to obtain, like gross energy intake (GEI) and CH_4 conversion factor (Y_m, the percentage of energy lost as CH_4 on total GEI); the Y_m to be chosen, among the ones proposed by IPCC, depends on the level of digestible energy (DE) and of milk yield typical of the country considered. However, Y_m depends on many factors like DMI, cow productivity, diet composition and dietary characteristics (Bannink et al. 2011), which makes not possible to explain the whole variability of this parameter only considering DE and milk yield. That is why IPCC Tier 3 seeks to apply the country-specific value of Y_m, validated by peer-review, to improve the precision of this estimation (IPCC 2019).

1.2.4. Mitigation strategies

The efforts to mitigate enteric CH_4 emissions have to be addressed to find strategies to reduce the environmental impact without penalizing animal productivity: the challenge is to fulfill SDG number 2 (provide food) and SDG 13 (urgent action) to combat climate change and its impact). Therefore, “dual-action” strategies, as defined by Arndt et al. (2021), are required to meet these two goals: it is not enough to consider CH_4 emission expressed in grams or litres per day; it is necessary to evaluate also metrics that take into account productivity parameters, like CH_4 yield (CH_4 per unit of feed intake) and CH_4 intensity (CH_4 per unit of milk produced). Improving animal productivity (through management, health, nutrition, and genetics) is, therefore, the first way to lower CH_4 intensity because CH_4 emission is diluted over a higher amount of product (e.g. milk). However, given that CH_4 intensity decreases curvilinearly with increased animal productivity, this approach is more effective for lower production/efficient animals, while it can be insignificant in the most advanced production systems (Beauchemin et al. 2020).

A variety of strategies can be implied to address directly or indirectly CH_4 emissions, and these strategies can be grouped into three main categories: animal and feed management, diet formulation, and rumen manipulation strategies (Table 1).



Animal and feed management	Diet formulation	Rumen manipulation
Feed processing	**By-products**	**Additives**
Forage processing	Fiber sources	Amino acids
Urea-treated straw	Soybean hulls	**CH_4 inhibitors**
Grain processing	Glycerol	**3-nitrooxypropanol**
Genetic Selection	**Lipid sources**	**Bromochloromethane**
Low residual feed intake	Extruded canola	Enzymes
Low CH_4 emitter	**Extruded linseed**	**Galactooligosaccharides**
Improving health	**Protein sources**	**Ionophores**
Improving pasture management	**Distiller´s dried grains with solubles**	**Monensin**
Grazing plus supplementation	Soybean meal	**Organic acids**
Grazing plus concentrate	Pulp sources	Carboxylic acids
Improved pasture	Citrus pulps	**Long chain fatty acids**
Increased Nitrongen fertilization	**Decreasing forage-to-concentrate ratio**	Unsaturated fatty acids
Legume-grass vs. grass only pastures	**Barley**	Docosahexaenoic acid
Reduced pre-grazing herbage mass	**Corn**	Probiotics
Increasing feeding level	**Minerals and Salts**	**Bacteria**
Increasing forage quality	**Oils and fats**	*Lactobacillus*
Decreasing grass maturity	**Canola oil**	***Propionibacterium***
Increasing corn silage maturity	**Coconut oil**	Yeasts
Optimizing temperature	**Linseed oil**	*Saccharomyces*
Total mixed ration feeding	Oil blends	**Secondary plant compounds**
Total mixed ration vs. grazing	**Rumen protected fat**	**Essential oils**
	Sunflower oil	**Essential oil blends**
	Tallow	Garlic
	Oilseeds	**Oregano**
	Canola seed	Flavonoids
	Cottonseed	Phenols
	Linseed	**Saponins**
	Sunflower seed	***Saponaria***
	Increasing protein	**Tea saponin**
	Tannifores forages	**Yucca saponin**
	Lespedeza	**Tannins**
	Leucaena	**Condensed tannins**
	Lotus	*Acacia*
	Sainfoin	*Quebracho*
	Urea	Hydrolysable tannins
		Defaunation
		Electron sinks
		Fumaric acid
		Nitrate
Strategies that decreased (adjusted $P < 0.05$) daily methane (CH_4) emission (g d^{-1}), CH_4 yield (CH_4 per unit of dry matter intake, g kg^{-1}), or CH_4 conversion factor [CH_4 energy (MJ) per gross energy intake (MJ), %] are presented in **bold**.		

Table 1: strategies to mitigate enteric CH_4 from ruminants. Source: Arndt et al. 2021.

1.2.4.1. Animal management

Genetic selection for lower-emitting cows has to deal with two main problems: moderate heritability and difficulty in measuring CH_4 in a manner that reflects the long-term phenotype of the animal, as asserted in the review of Beauchemin et al. (2020). However, improvement through genetic selection can be achieved if CH_4 intensity is considered because maintenance energy is a fixed cost and a function of body size, and CH_4 production is proportional to the energy intake of the cow (Knapp et al. 2014). Thus, selecting for increased milk yield without increasing DMI, or for reduced body size without reducing yields of milk and milk components, or for improved feed efficiency are good strategies to dilute maintenance energy costs of the animal (Knapp et al. 2014).

Early rumen manipulation appears to be more feasible because the microbial community of the newborn ruminant is more prone to manipulation, and thus it may be possible to direct it to the use of an alternative [H] sink. For example, Abecia et al. (2013) hypothesized that the early life manipulation performed on goats kids through the use of bromochloromethane favoured propionate metabolic pathway as alternative electron acceptors since a reduction in the ratio acetate:propionate was noticed in treated kids compared to untreated ones. However, persistence throughout adulthood has to be verified (Beauchemin et al. 2020). For example, Saro et al. (2018) found a decrease in CH_4 production at 8 weeks of age for lambs treated with garlic essential oil (EO) and lineseed oil since birth; however, this result was not confirmed at 14 weeks.

Another option is increasing the productive lifetime of the cattle since, as mentioned before, CH_4 can be considered a fixed energetic cost. Therefore, all the management practices aimed at improving the health and well-being of the animals (abatement of heat stress, reduction of disease incidence in the transition period, and others) can reduce methane intensity. In particular, improved fertility can strongly impact the lifetime since, given US as an example, almost 19% of culling was associated with reproduction problems (Hadley et al. 2006). Therefore, proper management of culling rate and the correct number of dry (and thus, the optimal length of the dry period) and replacements cows (and thus, proper age at first calving, low mortality, and morbidity rates) in the herd have the potential to mitigate CH_4 yield because they are the result of the effectiveness of the reproduction strategies. Even if other factors are taken into account (e.g. enough time in the dry period for the mammary tissue to involute and regenerate; culling rate to allow genetic progression of the herd; heifers old enough to sustain first calving and high lifetime production; beef production from dairy animals) the improvement in herd performances resulting from this optimal management can lead to lower CH_4 per kg of ECM of 9 to 19% (Knapp et al. 2014).

1.2.4.2. Diet

Diet manipulation can be highly effective, and it has the advantage of being a direct method, thus applicable in most systems (Beauchemin et al. 2009). This approach can be pursued through the choice of ingredients which can promote the alteration of VFA production patterns; through the increase of the rate of passage, which can alter microbial populations and VFA production patterns and shift some digestion to the intestines; through an enhancement of lactation performance promoted by better-quality diets, in order to reduce CH_4 intensity (Knapp et al. 2014).

1.2.4.2.1. Oil and fat supplementation

Dietary inclusion of oil and fat allowed a -20% reduction for CH_4 emissions and -15% for CH_4 yield, according to the meta-analysis of Arndt et al. (2021), and the effect may persist in the long term (Patra 2013). The potential of added fat was also studied by Beauchemin et al. (2008), who found that every 1% increase of supplemental fat in the diet corresponded to a reduction of 5.6% for CH_4 yield. This solution has other connected advantages because they are natural products, increase the energetic concentration of the diet, make the feeds less dusty and more flavoured, and, according to the fat source, change the fatty acid profile of milk (Toprak 2015). However, oil and fat addition caused a reduction of DMI and fibre digestibility, which can explain the positive effects on CH_4, together with the higher supply of non-fermentable, highly digestible energy and direct inhibition of methanogenesis by unsaturated fatty acids. Despite lower intake, the resuts of a recent metanalysis study showed that milk yield was not affected by fat supplementation (Arndt et al. 2021). Other modes of actions for CH_4 inhibition by added lipids are biohydrogenation of unsaturated fatty acids, especially the long-chain ones, which compete with methanogens for [H] use (Czerkawski 1972); enhanced propionic production, that again compete for [H]; direct protozoal inhibition (Johnson and Johnson, 1995). However, the recent meta-analysis of Dai and Faciola (2019) found that added lipids only numerically reduced protozoa number, while CH_4 yield was statistically reduced compared to control. In particular, the authors found that long-chain fatty acids (e.g. the ones contained in linseeds, rapeseeds, and sunflower seeds) did not affect protozoa number, while medium-chain fatty acid (e.g. the ones contained in coconut oil, or lauric and myristic acids) significantly reduced it (5.58 vs 5.97 log cells/mL of control). These findings highlight the importance of factors such as chain length, degree of unsaturation, the physical characteristics of the feed, amount and concentrations of oil and fatty acids, in affecting CH_4 emission and lactation performances (Toprak 2015). Possible detrimental factors, like increased feed cost and reduced digestibility, appear to be the main restrain for this solution, otherwise easy to apply in commercial farms (Beauchemin et al. 2020).

The most effective vegetable oils examined by Arndt et al. (2021) were coconut oil, rapeseeds oil, linseed oil, and sunflower oil; it also has to be underlined that the inclusion of oilseed achieved similar reductions to the ones found for oils (-20% for CH_4 emissions, -14% for CH_4 yield, and -12% for CH_4 intensity).

1.2.4.2.2. Forage and concentrate ratio and forage quality

As discussed above, two metrics to define enteric CH_4 impact from dairy cows are CH_4 production per kg of DMI and per kg of milk yield. Thus, decreasing these two ratios (diluting the grams of CH_4 on higher DMI or milk yield, respectively) could be an important strategy to mitigate this impact. Increasing feeding level is a controversial solution because it was associated with higher CH_4 emissions (g/d), +58%, but lower CH_4 intensity, -8% (Arndt et al. 2021), due to the high correlation of DMI with both CH_4 emissions (Mills et al. 2003) and milk yield (Hristov et al. 2005). To overcome these controversial results, higher DMI should be obtained through higher concentrate inclusion or high-quality diets.

Decreasing dietary forage-to-concentrate ratio achieved a reduction for CH_4 yield of -13% (Arndt et al. 2021), probably due to a shift in rumen fermentation patterns because concentrates are typically rich in starch, which lead to more propionate and butyrate than cellulose fermentation, competing with methanogenesis for [H]; furthermore, a decrease in rumen pH may inhibit methanogens, as confirmed, for example, in the experiment of Russell (1998). This solution led to higher DMI and milk yield as well, but it is important to evaluate if these improvements are counterbalanced with a higher cost of the ration and higher risk of subacute ruminal acidosis (Arndt et al. 2021). In the recent study of Ferris et al. (2020) on grazing dairy cows, the concentrate was fed at two different levels: 3 and 6 kg/d; thus, considering grass DMI on total DMI, the forage to concentrate ratio of the two diets was 83:17 and 67:33, respectively. The dietary treatment with 6 kg/d of concentrate significantly decreased CH_4 production per kg of ECM (11.4 vs 12.4 g/kg from cows fed 3 kg/d of concentrate) mainly because of an increase in milk yield (22.6 vs 19.6 kg/d). However, daily CH_4 production was not affected.

To achieve high levels of productivity reducing the quantity of concentrates, cows should be fed high-quality diets. These diets provide an adequate amount of energy and protein to meet the animal's nutrient requirements in terms of maximum productivity, good health, and reproductive efficiency (Weller et al. 2007). These diets allow higher feed efficiency and lower environmental impact per kg of milk also in terms of N excretion, besides improving farm self-sufficiency (Gislon et al. 2020b). A way to have high-quality diets, as mentioned before, is working on forage quality (i.e. higher NFC/NDF ratio and less lignified NDF). This can be realized with different strategies, such as the use of less mature forages, genetic selection for higher digestibility (e.g. brown midrib corn), or proper storage or ensiling (Knapp et al. 2014). In particular, decreasing grass maturity caused a reduction of -4% for CH_4 yield and -13% for CH_4 intensity due to higher milk yield caused by higher protein and energy digestibility (higher NFC and lower lignin concentration) associated with younger forages (Arndt et al. 2021). In Cabezas-Garcia et al. (2017), an increasing proportion of early-cut grass silage was used to partially substitute concentrate in the diet and the results showed that the higher quality of the early-cut silage, in terms of higher OM digestibility (OMD), counterbalanced the lower amount of starch in the diet; thus milk production and CH_4 emissions were not affected by the diet. On the opposite, in the work of Hatew et al. (2016), increasing corn silage maturity (i.e. from 25% DM to 40% DM) led to the reduction of CH_4 production (from 390 to 361 g/d). The authors explained these results considering the higher concentration of starch, the lower NDF, and the lower ruminal starch fermentation with the advanced maturity of corn silage; so, in contrast with grass, higher quality could be associated with later maturation stage for corn. In general, improving forage quality can have two side effects: higher ratio NFC/NDF and less lignified NDF, lead to higher availability of [H], as a consequence of improved OMD in the rumen; but greater DMI due to faster passage has the potential to reduce CH_4 yield and intensity (Beauchemin et al. 2020).

1.2.4.3. Rumen manipulation by feed additives

Rumen manipulation through compounds that can modify rumen fermentation to decrease methanogenesis is an active and challenging field of research, and this kind of investigation should be considered a high priority (Beauchemin et al. 2020). The ideal compound should directly inhibit methanogenesis both in a short time and persistently; not have toxic effects for animals, humans, and the environment; be cost-effective for producers, and possibly increase productivity and profitability.

1.2.4.3.1. Secondary plant metabolites

A broad category of rumen modulators is represented by secondary plant metabolites, or phytocompounds, which includes molecules like EO, tannins, saponins, flavonoids, and organosulphur compounds. These metabolites are not involved in growth, development, or reproduction (primary metabolism) in the plant, but carry out other important actions like protection from insect predation and microbial infection.

Essential oils. Different types of EO have been widely investigated, mainly in vitro, with positive results in mitigating CH_4 emission and modulating rumen fermentation due to their natural origin and safety. In particular, EO from thyme, oregano, cinnamon, and garlic showed the most consistent results (Cobellis et al. 2016b). Generally, EO are blends of different compounds, and their composition is highly dependent on factors like the plant species or organ (leaves, stems, flowers, fruits, roots), the environmental conditions where the plants grew, the physiological stage and age of the plant, and the extraction method (solvent, cold temperature under pressure, steam distillation) (Michalak et al. 2021). The single compounds contained in each EO are the bioactive molecules responsible for the modulation of ruminal fermentation, as some of these compounds have bacteriostatic or bactericidal properties. The mode of action seems to be due to their lipophilic nature and the ability to disrupt the cytoplasmic membrane causing increased permeability and leakage of the cell content (Hart et al. 2008).

An example of the great interest in EO is the meta-analysis conducted by Belanche et al. (2020) on the addition of a commercial blend of EO (Agolin® Ruminant), in the ration of dairy cows. This blend is composed of various plant species metabolites, like coriander (*Coriandrum sativum*) seed oil, eugenol, geranyl acetate, and geraniol. Including a total of 23 studies, the authors found that this blend decreased CH_4 for all the metrics considered (-8.8% for CH_4 production, -12.9% for CH_4 yield, and -9.9% for CH_4 per kg of ECM). Besides, milk yield was increased by 3.6% and dairy efficiency by 4.4%.

A possible disadvantage of EO is that, in some cases, their action is not specific on methanogenesis, thus causing undesirable adverse effects (e.g. impair digestibility) depending on the type of EO, substrate, and dose. The selection of the proper dose, in particular, is another critical point, as there is a narrow range of inclusion for the EO to be effective without detrimental effect (i.e. reduced digestibility) (Kholif and Olafadehan 2021). The problem of dosing EO was addressed by Khiaosa-ard and Zebeli (2013) in their meta-analysis. In the above mentioned study, different EO (e.g. from anice, garlic, juniper berry) bioactive compounds (e.g. cinnamaldehyde, eugenol, thymol, carvacrol), and their blends were included in the dataset. Only in vivo studies were considered, but for dairy cows, no effect on CH_4, VFA, DMI, and milk yield was dose-dependent, probably because the doses implied were low (between 0.01 and 0.43 g/kg diet DM).

Another drawback is the possible loss of effectiveness of EO in the long term, probably due to microbial adaptation (Klop et al. 2017), and long-term experiments are required to confirm the possible positive effects of EO (Khiaosa-ard and Zebeli, 2013). Among the disadvantages, impaired digestibility was found, for example, by Cobellis et al. (2016a), testing different combinations of EO selection. The authors obtained reduced DMD for 4 out of 5 combinations tested, compared to control, while for one of them (blend of EO from Ceylon cinnamon bark, dill seeds, eucalyptus leaves), only a numerical reduction was observed. Interestingly, in this paper, NDFD was not affected by the treatments. Most importantly, after in vitro screening, in vivo studies are required, and practical matters like variability of composition, low chemical stability, the requirement of a proper way of storage, palatability, possible off-flavors of the product, and cost, have to be further investigated before the application in commercial farms (Cobellis et al. 2016b). Finally, other beneficial aspects, like positive influence on gut microbiota and antioxidant properties (Akram et al. 2021), also deserve to be considered.

Achillea moschata essential oil. An EO that showed potential for methanogenesis inhibition is obtained by steam distillation of the dried aerial parts of the plant *Achillea moschata* Wulfen, a plant belonging to the Asteraceae family. The peculiar properties of this genus led to the farming of several *Achillea* species, grown to employ bioactive substances in different industries (Alsohaili and Sulaiman 2021). Furthermore, the genus *Achillea* has been the focus of recent research concerning food (Hashemi and Khodaei 2021), cosmetics (Shah et al. 2015), and pharmaceutical (Garcia-Oliveira et al. 2021) industries due to the bioactive compounds (mainly terpenoids and phenolic compounds) found in the EO of these plants, which confer antioxidant and anti-inflammatory activity (Açıkgöz 2020). Other important characteristics of *Achillea* are antimicrobial and antifungal activities. These properties have been widely investigated, implying EOs and plant extracts of different *Achillea* species, as reviewed by Salehi et al. (2020).

Achillea species have been used traditionally as ethnoveterinary medicines for ruminants to cure mastitis, wounds, and sternal abscesses (Lans et al. 2007). More recent is their use as rumen modulators, especially in the form of EO. Abdallah Sallam et al. (2011) evaluated the EO of *Achillea santolina* at different doses with in vitro gas production technique. They found that only the highest dose tested (75 µL in 75 mL of inoculum) reduced CH_4 per g of truly degradable organic matter compared to control, but this caused a reduction of the total gas produced at 24 h of incubation. No effect on CH_4 production and linear reduction in digestibility was also found by Kahvand and Malecky (2018) with increasing doses of *Achillea millefolium* EO at 24 h incubation. At long incubation time (i.e. 10 d), Demirtas et al. (2020) did not find any effect of *Achillea millefolium* extract on CH_4, protozoa number, total and single VFA production, but the treatment significantly reduced dry matter digestibility.

The reasons for the interest in the EO of *Achillea moschata* as rumen modulator are mainly three. First of all, it showed a broad-spectrum antimicrobial activity, at least comparable, if not better, to one of the conventional antibiotics, like erythromycin and ceftazidime (Vitalini et al. 2016). In Apel et al. (2021) study, *Achillea moschata* showed higher antimicrobial activity than *Achillea millefolium* for bacteria like *Staphylococcus aureus, Staphylococcus capitis,* and *Staphylococcus hominis.*

Secondly, the main pure compounds, as characterized by Vitalini et al. (2016), of this EO are camphor (CAM; representing about 27.2% of the oil), 1,8-cineol, also known as eucalyptol (EUCA; 10.7%), and bornyl acetate (BOR; 6.21%). According to Si et al. (2006), CAM and EUCA are known for their strong antimicrobial potentials. For example, they were considered the most potent antimicrobial agents in the study of Sökmen et al. (2004), where EO of *Achillea biebersteini* was evaluated in vitro. Furthermore, even if present in a lower amount in *Achillea moschata* EO, borneol is a valuable antimicrobial compound, as found by Abdossi and Kazemi (2016), who ascribed most of the antimicrobial activity *of Achillea millefolium* EO to borneol. However, in the above-mentioned study, borneol represented between 20.1 and 36.4% of the EO. Finally, two recent papers from Joch et al. (2016, 2018) demonstrated the potential of CAM and BOR in modulating ruminal fermentation. In the first paper (Joch et al. 2016), 11 individual active compounds of EO at a dose of 100 µL/L, including BOR, were tested within in vitro batch ruminal fermentation system for 24 h. The most effective compound was BOR because it reduced CH_4 emission, compared to control, i.e. fermentation bottles without additives (10.8 mmol/L vs 17.3 mmol/L), without decreasing VFA production (91.1 mmol/L vs 102 mmol/L of the control) neither changing the molar proportion of the single VFA. For this reason, the authors investigated whether also different concentrations of BOR (500 µL/L and 2000 µL/L) could be effective, using the same in vitro method. The previous results (reduction of CH_4 without decreasing VFA concentration) were confirmed for the tested doses. In the second paper (Joch et al. 2018), 7 pure compounds (among them CAM and BOR) were tested at 9 different doses. For CAM and BOR, the lowest concentration that decreased CH_4 production was 480 mg/L. However, with BOR, this concentration decreased total gas production as well. In the second experiment of this study, the lowest concentration that decreased CH_4 for each compound was tested again for possible changes in ruminal bacteria composition. Compared to control, both BOR and CAM reduced CH_4 production (-20.1 and -20.9%, respectively) without decreasing gas production. However, contrary to BOR, CAM reduced total VFA (84 vs 99 mmol/L of the control). Regarding bacterial composition, the addition of BOR and CAM caused an increase in the relative abundance of the phylum *Firmicutes* and *Proteobacteria,* while it decreased the *Bacteroidetes.* The authors concluded that BOR was confirmed to be the most beneficial compound for CH_4 mitigation. The other main constituent of *Achillea moschata* EO, EUCA, was tested as a pure compound with in vitro gas production technique in 16 h incubation experiment by Araujo et al. (2011). The authors concluded that EUCA had only minor effects on fermentation.

Tannins. Tannins are another class of plant secondary metabolites. As reviewed by Frutos et al. (2004), they can be found in a variety of plant species, and, especially in the most valuable plant components (e.g. new leaves and flowers); environmental distress factors (e.g. high temperature, water stress, extreme light intensities, poor soil quality) increase their concentration in plants. Tannins are polyphenolic compounds and they comprehend 2 major categories, condensed and hydrolysable tannins.

They showed a promising but variable response in decreasing CH_4 at low concentrations typical of many forages and feed supplements. Their mode of action has not been totally clarified (Aboagye and Beauchemin 2019), but different hypotheses have been made. Diaz Carrasco et al. (2017) found a reduction of rumen methanogens with tannins supplementation. Bhatta et al. (2009) found an effect on rumen methanogenic archaea and also on protozoa, both reduced by the treatments with tannins. Carulla et al. (2005) suggested that the reduction of CH_4 emission was due to tannin negative effect on NDFD, which altered the acetate:propionate ratio. Instead, Becker et al. (2014) found that catechin, a tannin precursor, can bind six hydrogen atoms; thus tannin might decrease CH_4 emission being an alternative H_2 sink.

In the meta-analysis of Jayanegara et al. (2012), CH_4 was reduced with increasing dietary tannins, with a quadratic response for in vitro studies and a linear for in vivo ones. However, this effect was associated with a reduction of OMD and, in particular, NDFD. Reduced digestibility is critical for their use in dairy cow feeding because their affinity to bind proteins and other compounds might also reduce DMI (Beauchemin et al. 2020). Lower palatability and conditioned aversions are other drawbacks of their inclusion in the diet (Frutos et al. 2004). On the other hand, their ability to bind feed proteins can increase rumen bypass and potentially provide a higher supply of metabolizable protein to the intestine (Aboagye and Beauchemin 2019). This was confirmed in the in vivo study by Focant et al. (2019), where the inclusion of 169 g DM of oak tannin extract in the diet of dairy cow reduced urinary N excretion of 12%. However, no effect on CH_4 was found.

Saponins. Saponins are high molecular weight glycosides, generally classified in two groups: steroidal and triterpenoid saponins (Kalinowska et al. 2005). These compounds affected methanogenesis, which was reduced both in vitro and in vivo (Dhanasekaran et al. 2020). Part of these effects could be due to saponins anti-protozoal activity, probably because of their ability to bind to the protozoal membrane and thus impair its functions (Wallace et al. 2002). This, in turn, is positive for microbial protein synthesis since saponins might prevent the predation of bacteria by protozoa (Patra and Saxena 2009). Guo et al. (2008) assessed that the reduction of CH_4 emission was due to the reduced activity of the microbial genes linked to CH_4 production. These authors found a reduction of the methyl coenzyme-M reductase subunit A (mcrA) (a crucial enzyme in the last step of the methanogenesis), but no difference in the total number of methanogens after the addition of saponins extracted from tea seeds in vitro. Patra and Saxena (2009) also suggested that saponins might change the site of digestion, depressing digestibility in the rumen and improving the one in the hindgut. This is unfavorable for rumen methanogenesis. In the meta-analysis of Jayanegara et al. (2014), increasing levels of saponin-rich sources (e.g. quillaja, gypsophilla, tribulus, tea, and yucca plants) in the substrate for in vitro incubation decreased CH_4 emission per unit of substrate or gas produced. This result was probably due to lower acetate and higher propionate production and reduction in protozoa count. In vivo, Mao et al. (2010) found a positive effect of tea saponins on CH_4 emission after 72 days of supplementation to growing lamb, not confirming, in this case, a possible long-term adaptation of the animals. Surprisingly, Guyader et al. (2017) found higher CH_4 production in dairy cows fed diets with 0.52% tea saponins inclusion. Furthermore, the authors found decreased DMI, milk yield, and dairy efficiency. These results were due to the failure of reducing acetate production and protozoa number by saponins: acetate increased (+6.2%), while protozoa number was unaffected.

1.2.4.3.2. Rumen defaunation

Rumen protozoa are microorganisms found to have a concentration of 10^3-10^6 across different ruminant species, i.e. sheep, dairy and beef cows (Dai and Faciola 2019). They represent less than 0.01% of microbial cells but between 5 and 50% of the microbial mass in the rumen (Williams and Coleman 1992). Another method that directly addresses rumen archaea is rumen defaunation. With either dietary manipulation, chemical compounds, or natural compounds, the aim is to reduce the number of methanogenetic archaea or protozoa in the rumen. However, as Broucek (2018) reviewed, several disadvantages exist: reduced digestion, difficulties for in-field applications, and only temporary positive effects because of adaptation and recovery of protozoa. In the meta-analysis of Li et al. (2018) it was found out that defaunation of rumen protozoa significantly reduced CH_4 emission, but its efficacy decreased over time, with CH_4 emission rising up of almost 0.5 L/week until week 12 after defaunation.

Protozoa. The interest in reducing protozoa number as a way to mitigate CH_4 derives from the fact that protozoa live in symbiotic association with rumen methanogens, with intracellular methanogens representing between 1 and 2% of the host volume (Finlay et al. 1994). In particular, through their hydrogenosomes, protozoa produce H_2, which is exploited by the methanogens for reducing CO_2 and thus obtaining their energy (Embley et al. 2003); H_2 removal is then beneficial for protozoa. This association is important in methanogenesis, as Finlay et al. (1994) found that ciliate protozoa-associated methanogen can account for up to 37% of total CH_4 production in sheep. Furthermore, Morgavi et al. (2010) found that the number of rumen protozoa could explain up to 47% of the variability in CH_4 emissions in the studies considered by these authors, and a reduction of this number of 10^5 cells/mL led to a reduction of 0.6 g of CH_4 yield. Guyader et al. (2014) assessed that protozoa concentration is even more important in determining CH_4 emission, as it can explain 93% of its variability.

Rumen protozoa are represented mainly by ciliates, and they belong to two orders: Entodiniomorphida and Holotrichs (Figure 5) (Williams and Coleman 1992).

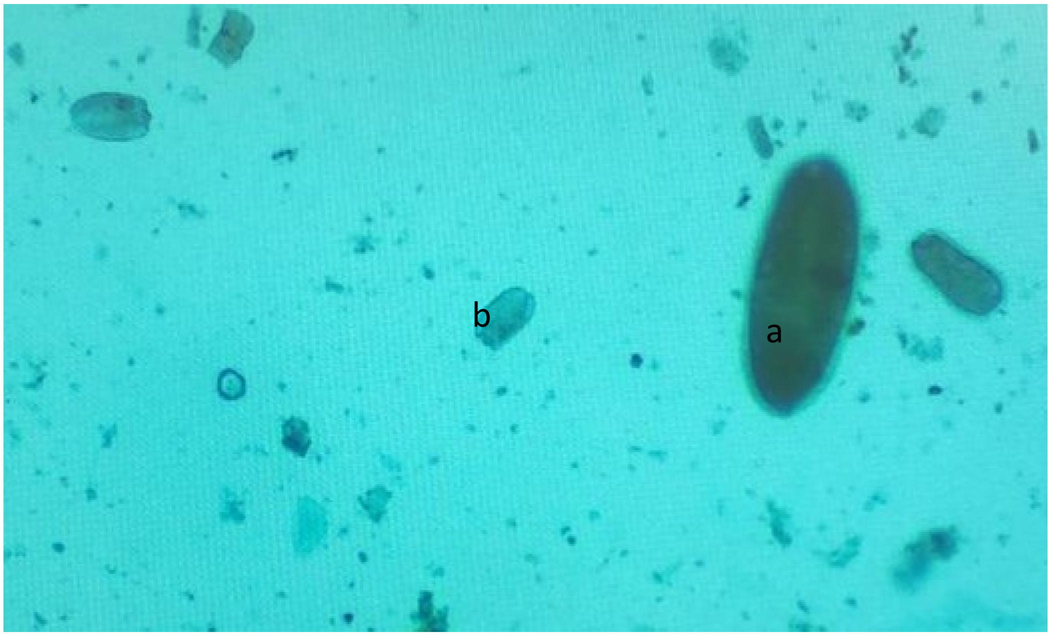


Figure 5: rumen protozoa: a) Holotrich b) Entodiniomorphid.

Belanche et al. (2014) demonstrated that large protozoa (Holotrichs) had a higher number of endosymbiontic methanogens and that Holotrich-associated community of methanogens had a different structure than one of the other protozoa, underling their key role in rumen methanogenesis. However, there was no difference in the concentrations of methanogens within protozoa and within the rumen as a whole. In another study from the same group of authors (Belanche et al. 2015), it was found that inoculating fauna-free sheep with different protozoal groups, Holotrich-associated CH_4 emission was not statistically different from emissions of fully faunated animals (49.5 vs 53.5 L/d). Furthermore, Holotrich-associated CH_4 emission per cell was 11.8 times higher than for total protozoa and 18.3 times higher than for Entodiniomorphids. These results suggested a major contribution from Holotrich in total CH_4 production.

In their book, Williams and Coleman (1992) described the biological roles protozoa carry out in the rumen, particularly bacterial turnover and fibre digestion. Protozoa predate other bacteria, engulf and digest them. As a result of this behavior, some products, especially amino acids, are not used by protozoa and are released in the rumen, where other bacteria can ferment them as carbon, nitrogen, and energy sources. In addition, protozoa leave the rumen more slowly than bacteria and this is why protozoa are associated with reduced outflow of microbial protein from the rumen. However, reduced total-tract digestibility of CP (-3%) was associated with defaunated animals (Newbold et al. 2015a).

In defaunated ruminants, total-tract NDF and ADF digestibility was decreased (Li et al. 2018), highlighting the role of protozoa in fibre digestion. Reduced NDF (-11%) and ADF digestibility (-9%) were found by Newbold et al. (2015a), as well. Protozoa appear to be involved in the initial stages of fibre colonization and digestion; thus, their elimination can negatively affect other fibrolytic microorganisms (Newbold et al. 2015a). Holotrichs are poorly active in fibre digestion, but they are able to ingest and store soluble sugars and starch grains, possibly preventing the onset of rumen acidosis (Williams and Coleman 1992).

These functions have been recently confirmed in the study of Williams et al. (2020). The authors found a range of glycosyl hydrolases and endo-1, 4-β-xylanases in the metatranscriptome of protozoa. Furthermore, pectin degrading enzymes were also found. Together with the high expression of cellulases and xylanases, these findings underlined the importance of protozoa in fibre digestion. A few proteases and lysosomal activity could explain protozoa predation of bacteria and fungi. Poor lipid digestion was confirmed by the presence of two lipases only; moreover, not highly expressed.

In the meta-analysis of Guyader et al. (2014), protozoa were positively associated with acetate and negatively associated with propionate concentration in the rumen. In contrast Li et al. (2018) reported that only butyrate was statistically higher in faunated ruminants than in defaunated ones, and the shift to propionate production was gradually lost over time, so much that defaunation eventually become disadvantageous in terms of energy supply for the animal. In particular, Holothrichs inoculated sheep were associated with 15% higher acetate, 57% higher butyrate, and 17% lower propionate concentration compared to fauna-free ones (Belanche et al. 2015).

1.2.4.3.3. Other compounds to manipulate rumen fermentation. Brief summary

3-nitrooxypropanol. Among rumen fermentation modulators, 3-nitrooxypropanol (3-NOP) proved to be the most effective one, achieving a reduction of -39% of daily CH_4 emissions, -37% of CH_4 yield, and -31% of CH_4 intensity, on average, without affecting DMI or milk yield (Arndt et al. 2021). This compound targets the nickel enzyme methyl-coenzyme M reductase, a fundamental enzyme in the methanogenesis pathway (Duin et al. 2016). The great advantage of 3-NOP compared with other additives is that its beneficial effect appears to be persistent. For example, in the recent study of van Gastelen et al. (2019), the addition of 3-NOP decreased CH_4 production by 15.8% on average compared to the control diet. No adaption mechanism was detected since CH_4 emission was not affected by the interaction treatment x days in milk during the 16 weeks-long dietary treatment trial. High specificity towards methanogens, minimum residues in milk and meat, and low safety risk are other features that reinforce the high potential of 3-NOP (Thiel et al. 2019a and 2019b).

Bacteriocins. Bacteriocins, like nisin and bovicin, are another category of compounds that proved to be effective in reducing CH_4 production. These are proteinaceous toxins produced by bacteria in order to inhibit other bacteria strains (Renuka et al. 2013). Further in vivo studies are required, though (Patra et al. 2017).

Probiotics. Probiotics are feed additives containing microorganisms that can provide a variety of beneficial effects to the host animal when included in the ration (Chiu et al. 2013). Some probiotics, like yeast, *Saccharomyces cerevisiae*, *Lactobacillus sporogenes,* and others, showed potential to reduce CH_4 production emission and intensity, with different modes of action, including enhanced propionate formation and improved animal productivity (Broucek et al. 2018). However, further studies are required because results have been either unsatisfactory, not conclusive, or have yet to be confirmed in vivo (Martin et al. 2010).

Alternative electron sink. Including electron sink into the diet (e.g. nitrate or organic acids), which can be an alternative to CH_4, also had positive results. In Arndt et al. (2021), they decreased CH_4 emissions of -17%, and CH_4 yield of -15%. Nitrate accepts [H] during its reduction to nitrite and then to ammonia. Furthermore, nitrate, or its reduced form nitrite, might also directly affect methanogen and protozoa (Lee and Beauchemin, 2014). Nevertheless, possible disadvantages of nitrate include the risk of toxicity for the animal, higher N excretion in the environment, and other undesirable end-products like N_2O (Beauchemin et al. 2020). Organic acids (malate, fumarate, acrylate, and others) are another alternative electron sink. They proved to be effective in vitro, but inconclusive or inconsistent results were observed in vivo (Broucek et al. 2018). Theoretically, malate and fumarate can reduce CH_4 because they are four carbons precursors of propionate, and so they stimulate [H] utilization when they are reduced to succinate (Song et al. 2011).

Ionophores. Ionophores deserve to be mentioned among rumen fermentation modulators despite being considered antibiotics, so their use is not allowed in Europe, where growth promoters and antibiotics other than coccidiostats and histomonostats, are not allowed as feed additives (OJEU, 2003). These compounds were initially used to improve feed conversion efficiency in ruminants, but they also showed potential for CH_4 inhibition. Thanks to their affinity with the lipid bilayer of the cell membrane, they facilitate ions movement across the cell membrane. Monensin, in particular, the most used compound belonging to this category (Tedeschi et al. 2003), is an antiporter with a high affinity for Na^+ (Russel and Strobel 1989). This action forces the microorganism to use their ATP storage to re-establish the ion gradient, leading eventually to cell energy depletion, impaired cell division, and death (Tedeschi et al. 2003). Chow et al. (1994) found that ionophores are effective against gram-positive bacteria and protozoa. Furthermore, they seem to select succinate- and propionate-forming bacteria, while inhibiting H_2-producing bacteria (Chen and Wolin 1979). Surprisingly, in the meta-analysis of Appuhany et al. (2013), monensin did not significantly reduce CH_4 production, nor Y_m in dairy cows, but had the effect of reducing DMI by 0.48 kg/d. The authors found that other factors, i.e. DMI and EE concentration, were more important in determining CH_4 production. These results are probably due to the low average dose of monensin across the dataset (21 mg/kg of DM) and to the long duration of treatment, which can lead to adaptation phenomena and overcome the positive effect of monensin over time (Broucek et al. 2018).

In conclusion, a variety of options for the mitigation of enteric CH_4 impact from dairy cows have been investigated. A combination of two or more of these anti-methanogenic strategies might be beneficial even if the effects are not fully additive (Beauchemin et al. 2020); in particular, combining inhibitors with complementary modes of actions can achieve the successful effect without using doses that are toxic or that causes detrimental effect for the animal (Patra and Yu 2013). Finally, other important factors have to be considered, like feasibility of implementation, economic impact, and regulatory policy (Knapp et al. 2014).

1.3. Dairy cow protein sources

1.3.1. Dairy cow diets and environmental challenges

Feed production is one of the main contributors to GWP derived from dairy cows. In Laca et al. (2020), this contribution was 20% and 40% of the total carbon footprint of 1 kg of fat and protein corrected milk for pastured-based and semi-confinement systems, respectively. According to the authors, the difference was due to different inclusion in the diet of feed produced off-farm (206 g vs. 648 g per 1 kg of fat and protein corrected milk, respectively). Similarly, in Lovarelli et al. (2019), purchased feed deeply affected all the environmental categories considered. In particular, purchased feeds contributed for the 19-33%, depending on the cluster of the dairy farm investigated, of total CO_2eq emission per kg of fat and protein corrected milk. The reason was due to the inputs used in feed production, like diesel fuel, fertilizer, and pesticides, and their transport to the farm. In addition, purchased feeds determined the 20-50% of freshwater eutrophication, the 29-62% of land use, the 24-59% of mineral, fossil and renewable resources depletion, and the 26-52% of photochemical oxidant formation.

Forages, like alfalfa, whole-plant corn, and grasses, are the main feedstuff in dairy cow diets (30-80% of total DM according to Gallo et al. 2013), and thus they are economically and nutritionally important because they maintain the rumen health. The remaining part of the diet can be represented by by-products (like distillers' grain or oilseed by-products), raw materials or concentrate mix, and feed additives, used to meet the nutritional requirements of high producing dairy cows (Eastridge 2006). Diets with high inclusion of grains and protein supplementation allow high digestibility, DMI and milk yield compared to diets based on forages only (VandeHaar and St-Pierre 2006). This is due to the dramatic increase in milk production in the last decades, and since DMI did not increase proportionally, diet density kept increasing to keep pace with the nutrients demand (Eastridge 2006). Additionally, feeds represent up to 50% of the operating costs for dairy farms in Europe, 70 % of which are for purchased feeds and 30 % for home-grown feeds (European Commission 2018).

A way to improve farmers' competitiveness and mitigate their environmental impact is to improve the resources efficiency, including feeds. Overfeeding less productive cows can increase the excretion of N and other nutrients in the environment, which is also an economical cost. N and P excretions cause eutrophication of water in lakes and streams. The N excreted is also in the form of ammonia, which is connected to haze, acid rain, and small diameter particulate matter (when converted into ammonium), or other volatile forms (N_2O, NO, NO_2) (VandeHaar and St-Pierre 2006). Furthermore, energy excess in the diet, typically in the form of starch, represents an extra cost, as well, for farmers and can lead to detrimental effects for health and production if subacute ruminal acidosis occurs (Moate et al. 2018). Therefore, precision feeding is a way to optimize the use of nutrients in the cow. Fischer et al. (2020) provided a feed allowance corresponding to the DMI of the 10% most efficient cows among a cohort of 68 heads to support this assumption. Feed restriction decreased DMI for less efficient cow of 2.6 kg/d, at a level of intake not different than the most efficient ones, without affecting milk production, body weight, or body weight change. Furthermore, strategies that optimize crude protein use efficiency (including the synchronization of protein and energy supply) have been shown to be a feasible way to reduce N excretion from cows (Børsting et al. 2003).

Overall, the choice of the feed ingredients to be used for TMR formulation is an important factor to optimize the sustainability of the dairy sector. Furthermore, since a high correlation between feed protein ingredients and environmental impact is observed, the possibility of using alternative protein sources to conventional soybean meals was evaluated in the present thesis.

1.3.2. Soybean meal: nutritional importance and main exporters

Soybean (*Glycine max*) is a versatile crop because it is consumed by humans as food, by animals as feed, and it is a vegetable oil source. In particular, soybean meal results from grinding the soybean cake after oil extraction, obtaining a high protein feed. The oil represents about 20% of the weight of the soybean, and the remaining 80% is crushed and destined for meal production. Soybean meal production represents 90% of the total soybean usage globally, and this is expected to be confirmed in the following years (OECD-FAO 2020) (Figure 6).

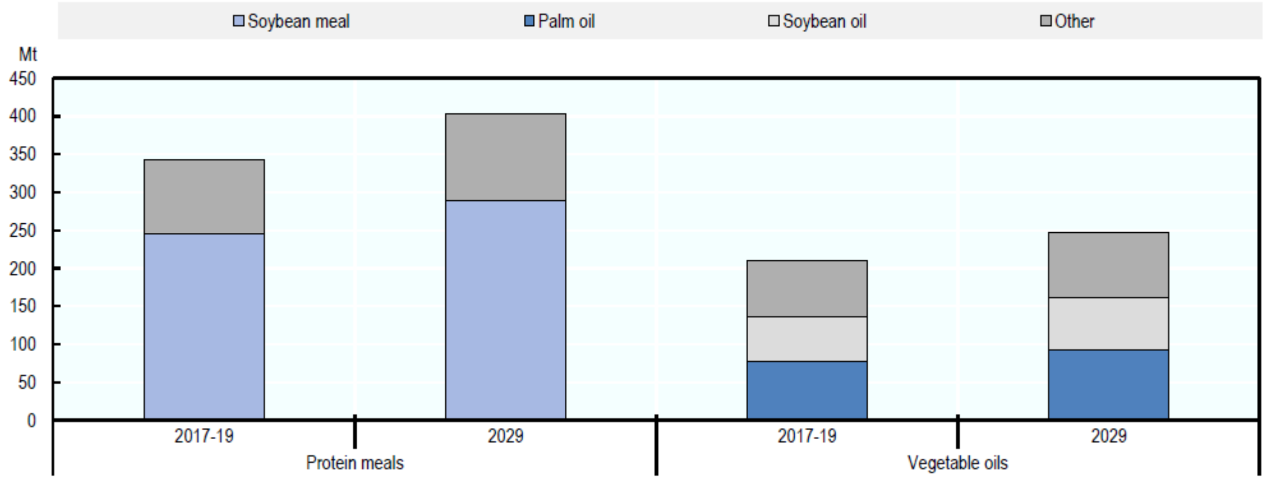


Figure 6: protein meal and vegetable oil production by type. Source OECD-FAO 2020.

Soybean meal is the primary protein source for livestock production worldwide, representing 69% of all protein sources used in animal feeding (Yildiz and Todorov 2014). It is sometimes considered the golden standard among protein sources because of the high protein and lysine concentrations (44-49% and 6.5%, respectively), moderate degradability of the protein, and high digestibility of RUP (NRC 2001). The digestible amino acid profile of soybean meal makes it the oilseed meal, which matches most the amino acids requirements of the animals (Yildiz and Todorov 2014). Due to its success, the production of this crop increased 7 times and the land occupied 4 times between 1970 and 2018 (Faostat.org data, elaborated by Malins 2020) (Figure 7), with over 50% of it produced in Brazil, Argentina and Paraguay in the year 2019 (Faostat.org).

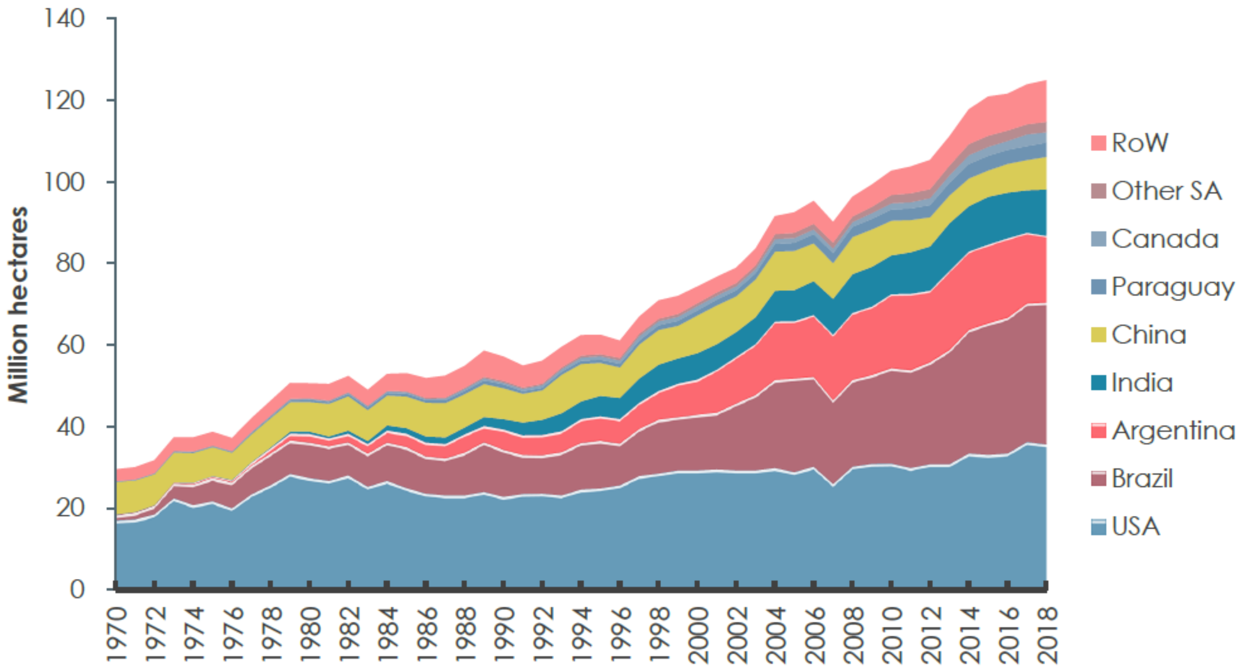


Figure 7: global soybean harvested area between 1970-2018. Source: Malins 2020.

Considering the data from comtrade.un.org, the import of soybean meal in the EU (with a conversion factor of 0.8 for soybeans and 1 for soybean cake) was about 30 million tonnes in 2020. Brazil was the main country of origin, with 49% of the total amount imported, followed by Argentina with 22% (Figure 8).

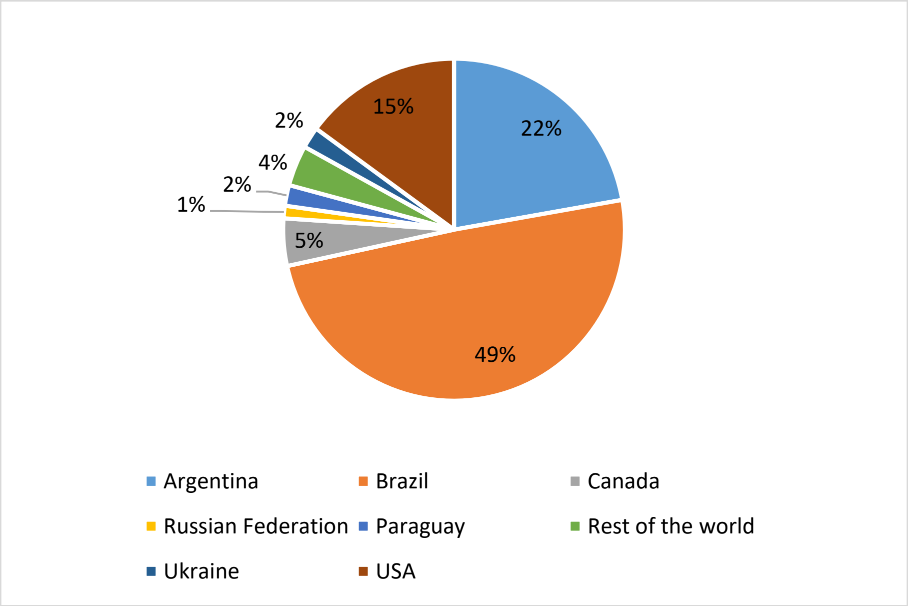


Figure 8: EU soy imports by country in 2020. Source: comtrade.un.org.

In the same year, Italy imported 3.5 million tonnes of soybean meal, with Argentina (39%) and Brazil (30%) as the main country of origin (Figure 9).

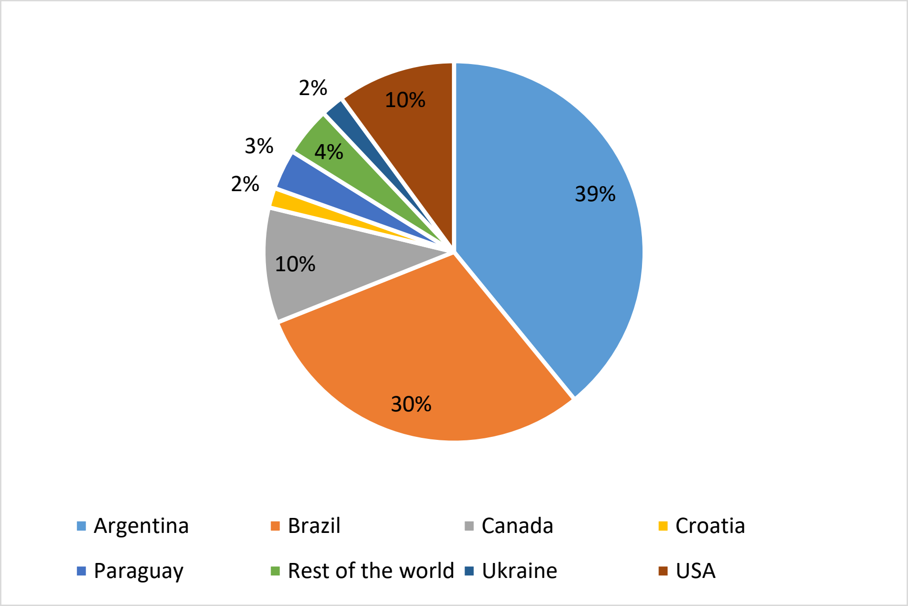


Figure 9: Italian soy imports by country in 2020. Source: comtrade.un.org.

1.3.3. The link between soybean meal and land use change

In the recent study of Escobar et al. (2020), it was estimated that the EU has the largest carbon footprint per unit of imported soybean equivalent, equal to 0.77 t/t, mainly due to LUC (more than 50%). "Land use" indicates the management regimes imposed on a specific site. If anthropogenic land use (e.g. agriculture, urban use, pasture) expands at the expense of natural vegetation, different environmental drawbacks arise, like CO_2 emission, change in biodiversity, soil erosion, and degradation (Dale 1997). Land use has historically changed according to changes in human needs due to economic, societal, technological, political, and environmental reasons. However, the actual rate of change is threatening the environment due to the rapidity of this process (Rounsevell and Reay 2009). According to the definition of Olson (2010), when the CO_2 is transferred from the atmosphere into the soil organic matter (humus) in the form of plants, plant residues, and other organic solids, we can talk about carbon sequestration. Instead, carbon storage is an increase in soil organic carbon stock over time, but not necessarily due to a net removal of CO_2 from the atmosphere (Chenu et al. 2019). Therefore, storing carbon in the soil for a long time can help mitigate GHG emissions. On the other hand, the soil organic matter with shorter residence time is essential for life because it is involved in the physical properties of the soil, like aggregate stability (Angers and Mehuys 1989); it is involved in soil fertility and soil biodiversity, as it is a trophic source for many organisms (Chenu et al. 2019). When land is cleared for agricultural production, carbon loss occurs on three levels: from the above-ground biomass, from the underground biomass, and from soil stocks. In each biome, the relative incidence of these losses may vary, depending on the most extensive carbon reservoir (i.e. with the aboveground biomass for the forest or soil carbon stock for the grassland). Furthermore, soil disturbance, like during tillage, is generally considered to increase soil organic matter mineralization due to the disruption of soil aggregates and the consequent release of CO_2 (Chenu et al. 2019).

In the data elaborated by Cuypers et al. (2013), soybean expansion was considered the driver of 19% of deforestation caused directly or indirectly by agriculture production between the years 1990-2008, with Brazil (65%) Argentina (19%), and Paraguay (7%) as the main countries where this phenomenon took place. The reason for this trend is that soy grows well in tropical climates, and the high demand for this commodity led countries like Brazil, Argentina and Paraguay to expand the land dedicated to soybean, even if this meant clearing high carbon stock and biodiversity of tropical forest (Figure 10).

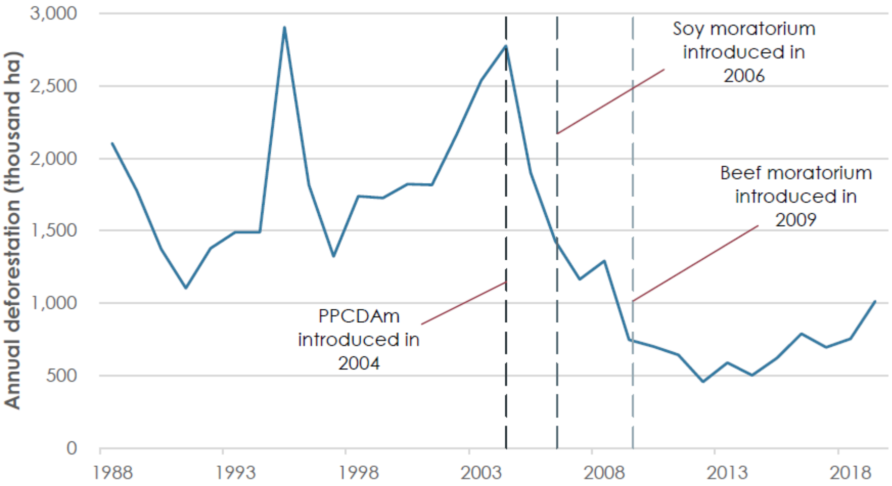


Figure 10: estimated annual Amazon deforestation between 1988-2018. Source: Malins 2020.

However, sometimes, the link between soybean cropping and deforestation is hard to establish because a few years (3 to 5 according to van Berkum and Bindraban 2008) between deforestation and soybean cropping may occur. For example, common management uses the new land as pasture for grazing cattle and then for dry rice cropping. However, the long-term aim of this management is to provide beneficial agronomic conditions for the subsequent cultivation of soybean. As a result, the public opinion and different stakeholders led the European importers to ask for a guarantee of higher sustainability in the supply chain of this crop. In 2006, this led the Brazilian Association of Cereal Exporters and the Brazilian Association of Vegetable Oil Industries (responsible for 90% of all soy purchased in Brazil) to sign the Soybean Moratorium. According to this agreement, soybean grown on former Amazon forest was banned, and some stakeholders received the assignment of monitoring the compliance to the Moratorium (Massoca and Lui 2017). Despite still being debated, this was a turning point because, since then, soybean expansion took place almost exclusively into former pastureland. However, a recent document of the European Commission (2019) stated that 8% of the soy land expansion took place into former forest land worldwide, making soy the second crop in this ranking after only palm oil since 2008. Authors like Arima et al. (2011) underlined that clearing pasture for soybean cropping can be considered Amazonian deforestation driven by soybean because it forces pasture to expand elsewhere in the forest. It is also debated if soybean expansion occurs in lower regulated areas, like the Cerrado in Brazil and the Gran Chaco between Bolivia, Paraguay, and Argentina; these are considered other hotspots for soybean expansion at the expense of the forest in South America. The Cerrado in particular, “the new deforestation frontier” (Malins 2020), is rich with biodiversity and comprehend different biomes (from grassland to forest) (wwf.panda.org), and it is also characterized by high levels of carbon stock, as reviewed by De Miranda et al. (2014).

The problem of deforestation of legal Amazon is still existing, and in the years 2019 and 2020 it has reached a new pick, outreaching 10 000 km^2 for the first time since 2008, in both years (PRODES data). Furthermore, other important factors are involved in this scenario besides the impact on biodiversity (Figure 11) and natural ecosystems balance, like the threats to traditional farming, and indigenous land rights, without underestimating the global demand for agricultural intensification (Graesser et al. 2015).

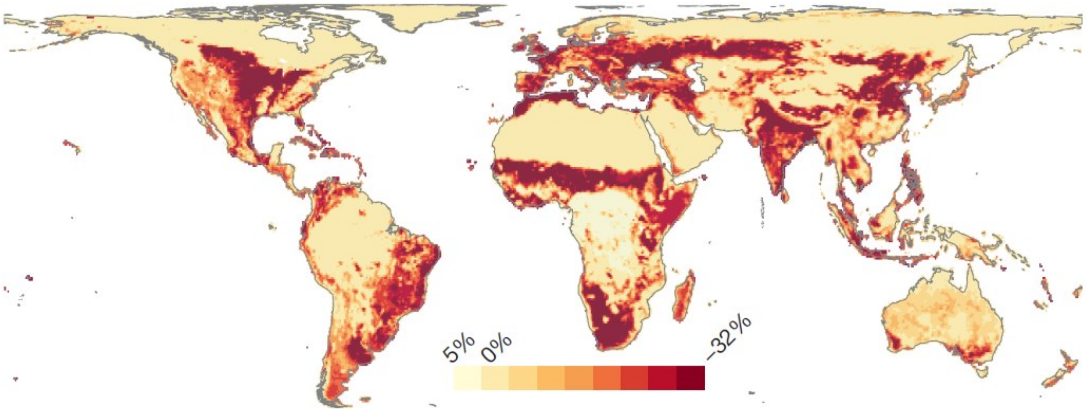


Figure 11: net change in local richness caused by land use and related pressures by 2000. Source: Newbold et al. 2015b.

1.3.4. Possible solutions to the use of conventional soybean meal

The use of feeds alternative to soybean meal is advised given the environmental problems (i.e. LUC) connected to the conventional production of soybean meal in the last decades. In order to replace soybean, other high protein content feedstuffs are required, like pulses or other oilseed meals. Below, the main advantages and disadvantages of these options will be briefly presented, without discussing in depth the complexity of protein feeding and nutrition in dairy cows. The focus will be, in particular, on soybean silage and responsible soybean meal, two feedstuffs investigated in the experimental part of the present PhD project.

1.3.4.1. Grain legumes

Grain legumes, like lupin, pea, and faba bean, could represent an alternative protein source that can be grown in cool temperate climate conditions. They belong to the family of Leguminosae, and are primarily cultivated for their protein-rich grains, which can be used for human or animal consumption (GL-Pro 2005). On average, the seeds from these species have a CP concentration of 25-30%, while oil concentration is less than 2%, differently from oilseeds, which contain at least 20% oil at maturity, and 20-50% CP after oil extraction (Ranalli 1995). Grain legumes can be considered as a source of energy as well, because of their high starch concentration (47.8–53.4% for pea and 42.2–45.1% for faba bean) (Aumiller et al. 2015), even if total-tract starch digestibility was lower than cereal starch in Larsen et al. (2009). Proteaginous seeds from grain legumes have been studied in the meta-analysis of Mendowski et al. (2021). The authors focused in particular on faba bean (30.8% of CP, on average in their dataset), lupin (36.1% CP), and pea (24.8% CP) vs soybean meal (50.3% CP). Due to the higher CP content and lower rumen digestibility of protein, the amount of protein reaching the intestine was at least 2 times higher for soybean meal compared to the proteaginous seeds considered (208 g/kg DM vs 113 g/kg of lupin, which was the best seed considering this parameter). Heat treatments of the seeds (i.e. with or without pressure, and others) helped reduce the gap with soybean meal in terms of CP ruminal degradability, with the strongest effect for autoclaving (i.e. cooking the seeds under high pressure). Considering the in vivo studies of this meta-analysis, none of the alternative protein sources performed as soybean meal, despite high variability in the results.

Lupin. Mainly three species of lupin are grown as animal feed: white, blue, and yellow lupin. In a recent study (Kuoppala et al. 2021), blue lupin supplementation in a diet based on grass silage decreased DMI compared to a diet with faba bean; however, considering these two supplements together, the intake was not different compared to cows fed a diet with canola meal. Milk yield was not affected, but the treatment with canola meal had higher milk protein concentration compared to lupin and faba bean. A positive environmental effect of lupin was found in Bryszak et al. (2020), where the authors found that the addition of lupin seed meal (100 g/kg diet) to the diet decreased CH_4 emission in vivo compared to the control diet (i.e. with a higher inclusion of soybean and canola meals).

Pea. Pea is a peculiar protein source because it is a protein and starch-rich feed. For example, the ground field pea used in Pereira et al. (2020) study had a protein and starch concentration of about 20% and 50%, respectively. The authors tested soybean meal or canola meal as companion rumen-degradable protein sources in a diet with 25% DM coming from field pea and about 12%, on average, from either soybean or canola meal. Pea and canola proved to be a better combination as a protein source since all the essential amino acids were increased in the plasma of cows fed this dietary treatment, as well as DMI, milk yield, and milk N efficiency.

Faba bean. Faba bean is another pulse rich in both starch and protein. Cherif et al. (2018) tested faba bean in the diet of dairy cows, with faba bean having about 30% of both CP and starch on DM basis. For this reason, three isonitrogenous and isoenergetic diets were formulated, including 17% of faba bean on total DM, in complete substitution of soybean meal (9% in control) and partial substitution of corn meal (- 8% compared to the control). No effects on DMI, milk yield and composition were found. Furthermore, the diet did not affect CH_4 production.

1.3.4.2. Other oilseed meals

Another option is the substitution of soybean meal with other oilseed meals like sunflower and canola meals. *Sunflower meal*. Depending on the degree of dehulling, sunflower meals can have protein concentrations between 23% and 39%, with high rumen degradability (78%, according to Yildiz and Todorov 2014). The amino acid profile is characterized by a high content of sulfur-containing amino acids like methionine but a poor concentration of lysine. In agreement with this profile, Drackley and Schingoethe (1986) found that a blend of soybean meal and sunflower had the most desirable amino acids profile among the ones tested in terms of mammary gland requirements. Other positive features of this meal are good palatability and lack of antinutritional factors. Instead, when sunflower meal had been used to replace soybean meal, cow performances were impaired. For example, in Oliveira et al. (2018), increasing inclusion of non-decorticated sunflower meal (from 0 to 21% of diet DM), in substitution of an increasing amount of soybean meal (up to complete exclusion from the diet) linearly reduced milk production and quality (i.e. fat and protein concentration). The authors ascribed these results to the low fiber digestibility of sunflower meal hulls, suggesting that dehulling might be advisable to increase the nutritive value of this meal.

Canola meal. Canola meal is another by-product of oil production, extracted from one of the two cultivars of rapeseed, also known as double-zero or double-low oilseed rape. Canola meal can contain between 32% and 40% CP on DM, with low rumen degradability (69% according to Yildiz and Todorov 2014) and higher concentration of methionine but lower of lysine compared to soybean meal. The ratio of lysine to methionine is about 3, resembling the one of milk protein, and this makes canola meal a valuable protein source for the requirements of dairy cows. Spörndly and Åsberg (2006) found high palatability of canola meal, testing different concentrate components in short terms experiments: heat-treated canola meal was among the preferred feed. Several studies evaluated canola meal in vivo with positive results. According to the elaboration made by Yildiz and Todorov (2014) the mean milk production response was 1 kg/d higher for canola meal compared to soybean meal. Besides, canola meal has a good sucrose content, found to be on average 6.1% in the study of Adewole et al. (2016), which is beneficial for microbial protein synthesis; furthermore, antimicrobial factors are low. Given the number of studies investigating canola meal in dairy cows, a few meta-analyses were conducted. In the one realized by Martineau et al. (2013), canola meal inclusion ranged between 5% to 17% of diet DM. A positive response was found for DMI and milk yield, when canola meal substituted another protein source in the diet, while the response of ECM, milk protein percentage, and milk protein yield were positive only when canola was used in substitution of a protein source other than soybean meal. This leads the authors to conclude that canola meal is a protein source at least as good as soybean meal. In another meta-analysis on the topic (Huhtanen et al.

2011), instead, canola meal was compared solely with soybean meal. In this work, the authors found higher responses than the ones found in Martineau et al. (2013). Despite the environmental concerns regarding the use of imported soybean meal, Lehuger et al. (2009) found that a ration based on soybean meal is more sustainable than one based on home-grown canola meal as protein source, in France. This unexpected result was due to the high impact of crop production, which is lower for soybean because this crop does not require N fertilization. It is also important to point that, in this study, LUC was not considered. Finally, Lage et al. (2021) compared canola meal (17% of diet DM) with soybean meal (either extruded or solvent extracted; 14% of diet DM, on average) also in terms of methane emissions. The authors found no effect on feed efficiency, ECM, and milk yield components, despite higher DMI intake for the diet with canola meal (27 kg/d vs 25 kg/d on average for the two soybean meal diets). Methane per kg of DMI was lower for canola meal, but no differences were found when CH_4/ECM or CH_4 in g/d were considered.

Dried distillers' grains. Dried distillers' grains are a by-product of the ethanol industry characterized by high protein concentration (45.8% for wheat dry distilled grain in Gibb et al. 2008, as an example) and reduced rumen degradability due to the heat treatment they undergo (56%-62% depending on the type of grain used, Yildiz and Todorov 2014). Lysine is low, but the ratio lysine:methionine is close to 3. In the vivo experiment of Garnsworthy et al. (2021), wheat-dried distillers' grains with solubles have been included in the different ration in the diet of early and mid-lactation cows, in substitution of increasing percentages of soybean meal. In the first experiment, only the diet with the highest inclusion of dried distillers' grains (24% of diet DM) decreased DMI, milk yield, and protein yield, but not milk protein concentration, compared to the other diets (0%, 8%, and 16% of inclusion of dried distillers' grain). In the second experiment, no differences were found in the productive response of cows fed 4 diets with 0%, 7.5%, 15%, and 22.5% DM coming from dried distillers' grain.

Responsible soybean meal. As mentioned above, the main environmental impact of soybean meal is due to the link between soybean cropping and LUC in South America. The data shown in Agri-footprint.com supported this assumption: the GWP of Brazilian soybean and soybean meal was much higher when considering LUC (5.60 and 4.67 kg CO_2eq/kg of product, respectively) than when LUC was not included in the environmental assessment (0.35 and 0.54 kg CO_2eq/kg of product, respectively). Thus, a soybean meal characterized by higher environmental sustainability could be regarded as an oilseed meal alternative to the conventional soybean meal. The importing countries know the importance of dealing with the environmental cost of soybean meal and started encouraging sustainable production and sourcing of soy. For these reasons, several initiatives arose in many European countries, including the demand for deforestation-free soy in the supply chains and the constitution of multi-stakeholder soy roundtables. In particular, the European feed industry association (FEFAC) defined as "responsible soy cultivation" a soybean whose production followed FEFAC Soy Sourcing Guidelines. The FEFAC guidelines of 2021 require several sustainability criteria in order to define "responsible" soybean production. Concerning environmental impact, FEFAC requires that the expansion of soybean cropping land complies with national laws and natural area preservation (reserve or conservation areas, riparian vegetation, flood plain, and wetlands). Thus, soybean produced in lands coming from illegal deforestation can not be defined as responsible. Furthermore, other sustainability requirements are demanded by FEFAC, like proper working conditions, implementation of good agricultural practices, and the protection of community relations. Not only LUC, in fact, but also other impact categories could be mitigated by a more sustainable soybean cropping, as achieved by the 14 farms participating ProAgros frames. These farms adhered to the Sustainable Farming Assurance Programme, aimed at producing commodities, like soybean, responsibly (sustainableassurance.com 2021). Soybeans and soybean meal produced in this way had reduced terrestrial acidification (0.0007 and 0.001 kg SO_2eq/kg of product, respectively), compared to the conventional way of production (0.003 and 0.003 kg SO_2eq/kg of product, respectively); human non-carcinogenic toxicity was reduced from 2.28 and 1.81 kg 1,4-DBC eq/unit of product for soybeans and soybean meal, respectively, to 0.88 and 0.70 kg 1,4-DBC eq/unit of product. As a result, GWP of soybeans and soybean meal produced responsibly was 0.27 and 0.39 kg CO_2eq/kg of product, respectively (agri-footprint.com).

The agreement FEFAC promoted across top European feed companies was that 100% of the soybean purchased would be responsible by 2025. According to the European Soy Monitor report (IDH 2020), in 2018, 22.5 million tonnes of soybeans were produced in compliance with FEFAC guidelines worldwide. This corresponded to 3.1 million tonnes more compared to 2017. From the total amount, 14.2 million tonnes (equivalent to 11.3 million tonnes of soybean meal) were destined to Europe. According to these elaborations, 38% of the soybean consumed in the EU in 2018 was FEFAC compliant. However, out of 14.2 million tonnes, only 7.2 million could be certified deforestation-free because only illegal deforestation is not in compliance with FEFAC guidelines, but FEFAC still accepts legal deforestation. Considering Italy alone, 36% of soybean meal consumption was FEFAC guidelines compliant and 20% deforestation-free in 2018 (IDH 2020). Another association that developed certification criteria for responsible soy production is the Round Table on Responsible Soy (RTRS). This association was founded in 2006 to provide the best knowledge for sustainable soy policy. Since then, it has promoted the production, trade, and use of responsible soy among different stakeholders at different levels of the supply chain. In 2014, the RTRS-certified production of soy was 1.4 million tonnes, representing less than 1% of the global production; Brazil (50%) and Argentina (33%) were the first countries for extension of certified soy area (Garrett et al. 2016). According to RTRS, between 2013 and 2020, the certified volume of responsible soy increased 4.5 times (4.7 million tons in 2020), with land implied for its production increased 3 times (1.2 million hectares in 2020) (Responsiblesoy.org).

As mentioned above for enteric CH_4 mitigation strategies, a variety of other factors (feasibility, economical, and political) has to be taken into account when considering the choice of a protein source. Responsible soybean meal could also be used to produce milk destined for Parmigiano Reggiano cheese in Italy. This cheese is a Protected designation of origin (PDO) product, and, as every PDO, it has to follow strict rules of production defined by the producer association. For the production of milk for Parmigiano Reggiano, cows have to be fed a diet containing at least 50% DM from hays, while silages are not allowed (Consorzio del Formaggio Parmigiano Reggiano 2021). Canola meal and distillers' grain, two of the protein source discussed above, are not accepted as well by the producer association. Milk destined for PDO cheese production has great economic importance in Italy, in particular. In 2020, 6.4 million tonnes of milk were destined for the production of PDO cheeses, representing 45.5% of the total milk available (national production + import) in Italy (clal.it). This amount of milk corresponded to a total of 488000 tonnes of PDO cheeses produced in Italy in 2020 using cows' milk. Parmigiano Reggiano was the second PDO cheese for the amount produced (144700 tonnes) and for economic value (1556 million euros) (ISMEA 2020).

1.3.4.3. Self-produced forages

Producing and feeding high-quality forages could be seen as a solution for protein supply to the farm. The forages most studied and used worldwide for dairy cows will be briefly presented below, focusing on the Italian situation. As mentioned, great importance will be given to soybean silage, whose use as a protein source was investigated in this PhD thesis's experimental part. Home-grown, highly digestible forages or fresh herbage cuts also have positive environmental (see the chapter about enteric methane above) and economic benefits. However, in Italy, most dairy farms are only specialized in corn silage production due to its high energy content, and it can account for up to 90% of the forages in the diet. This leads almost to energy self-sufficiency, considering the requirements of the cows, but to a deficiency of protein. The CP requirements are addressed buying either row materials (like soybean and/or canola meal), coming from overseas, or concentrate mixes, accounting for up to 55% of diet DM (Borreani et al. 2013).

Corn silage. Due to its high agronomic yield, whole-plant corn silage is the most popular forage used worldwide in dairy cow diets (Ferraretto et al. 2018). However, its chemical composition made it a peculiar forage, as corn silage provides physically effective NDF (mainly in the stover) besides a high amount of starch and thus energy (with the kernel), as, at physiological maturity, about 50% of DM is forage and the other 50% is grain. When corn reaches a DM concentration of 25-30%, it can be easily harvested and stored as silage (Ferraretto et al. 2018; Klopfenstein et al 2013). In the meta-analysis of Ferraretto and Shaver (2015), the average DM, NDF, and starch contents for conventional corn silage were 33.9%, 42.8%, and 29.7%, respectively, while ruminal NDFD was 46.7% of total NDF. According to Klopfenstein et al. (2013), the forage content of US dairy cow diets can represent 40-60% of diet DM and corn silage can be up to 100% of this share. In Italy as well, more precisely in the Po Plain, corn silage is the most popular forage in dairy cow farms, used in more than 90% of the farms (Figure 12) as a TMR ingredient and with inclusion in diet DM mostly between 30 and 36% (Gislon et al. 2020a).

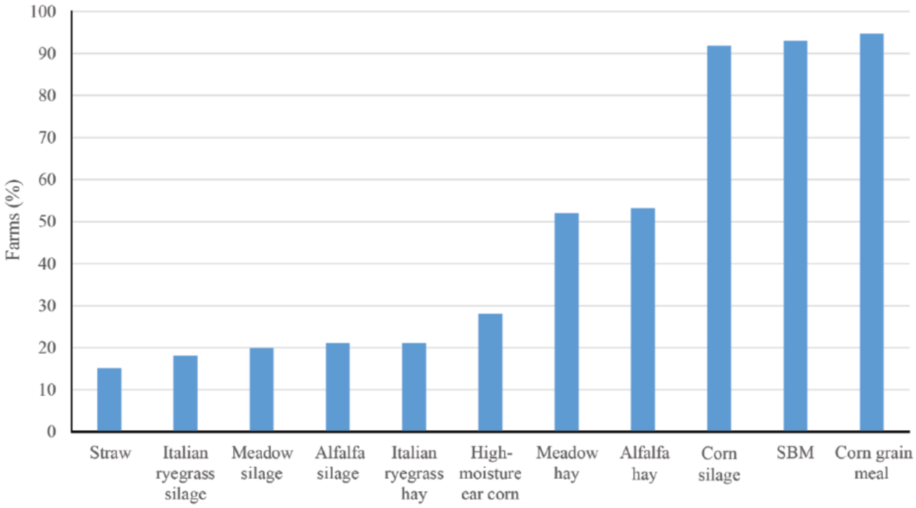


Figure 12: utilization of main ingredients (% of farms that use the feed) for lactating cow TMR formulation. Source: Gislon et al. 2020a.

In another study in the same area, it was found that corn silage was grown in about 83% of the farm as a single crop and in 54% as a double-crop; the average yield was 19 t DM/ha, with an average NDF and CP concentrations of 42% and 7.9%, respectively (Zucali et al. 2018).

However, corn has high inputs requirements in nitrogen fertilizers, agrochemicals, and non-renewable energy sources, while other forages can be competitive with corn silage. Given the environmental and economic concerns explained above, Tabacco et al. (2018a) evaluated an alternative forage system in North Italy, aimed at increasing the protein self-sufficiency of the farms, and thus reducing the reliance on soybean meal and other protein concentrates, as well as at decreasing the use of feed potentially suitable for human consumption. This system was based on forages with high energy and protein content, obtained with early cutting, preserved as silage, legumes cropping, and double cropping: Italian ryegrass, meadow, and alfalfa silages were all increased compared to conventional forage system, at the expense of hays and partly replacing corn silage. The authors found that DM, CP and ME yield per hectare increased, but purchased inputs were reduced.

Grass silage. Grass silage is traditionally a popular forage in Europe, New Zealand, Australia, and North America, especially in the winter (Keady et al. 2008). In the temperate areas, like North Italy, grass is more popular as hay: Gislon (2020a) found that meadow hay was fed in about 50% of the farms, while meadow silage and italian ryegrass silage in about 20% each (Figure 12). Ryegrass, in particular, was found to have 57% NDF and 5.5% CP, with a yield as hay of 6.4 t DM/ha, in this area (Zucali et al. 2018). Patterson et al. (2021), in a 20 years review, found that the CP concentration of Northern Ireland grass silages ranged, on average, from 11.8% of the first cut to 14% of the third cut. A great part of this protein is rapidly degraded in the rumen (van Vuuren et al. 1990). The high quality of the fiber of ryegrass was addressed in the study of Hoffman et al. (1998), for example, finding that perennial ryegrass, harvested at boot stages of maturity, had a potentially digestible NDF of 39.1%, higher than alfalfa, harvested at late bud stage, with 30.3%. Pirondini et al. (2015) found that ryegrass silage had the highest in situ NDFD among the forages tested (alfalfa silage and hay, con silage, and ryegrass hay), at 24 (54.7%), 48 (75.1%), and 240 (88.1%) h. These results were confirmed in vivo in Gislon et al. (2020b) study, where a diet with 19.1% of DM coming from ryegrass silage had the highest NDFD (47.4%), together with a diet based on corn silage. Higher NDFD favors higher DMI. This was confirmed in the study of Bernard et al. (2002), where cows fed diet with increasing inclusion of ryegrass silage, in substitution of corn silage, increased their NDF intake linearly.

Pasture. Pasture can be considered as another high-quality forage source of protein if adequately managed. Grazing systems are still used, especially in countries where the temperate climate allows more extended exploitation of pasture. For example, in Australia, in 2021, 74% of the herds grazed for 12 months (Dairy Australia 2021), while in Ireland, fresh grazed herbage is the main forage of the diet from March to October (O'Brien et al. 2018). In Italy, pasture-based farming is a minor reality on the total milk produced nationally (Borreani et al. 2013). This feeding system proved to be economically advantageous, particularly when a higher percentage of total diet DM comes from pasture rather than concentrate (Hanrahan et al. 2018). High-quality pasture is a CP-rich forage: just as examples, CP concentration in Waghorn et al. (2016) was 26.5%; in the two dietary treatments of Wims et al. (2010), it was 27.5% and 21.1%. However, pasture can be high in rumen-degradable protein, so it has to be verified if enough digestible protein reaches the intestine (Tamminga 2006). Doran et al. (2021) tested two different rates of protein supplementation for grazing cows, 13% and 18%, but assuring the same level of PDI. However, milk yield and protein yield decreased for the cows fed a low-CP supplementation, even if milk composition was not affected.

Alfalfa silage. Tabacco et al. (2018a) highlighted the importance of legume forages. From an agronomic and environmental standpoint, legumes provide several benefits like low use of fertilizer, due to nitrogen fixation in the soil, which results in lower GHG emissions; increased diversification in the crop rotation, with a related increase in below- and above-ground biodiversity, which changes weed, pest and disease pressure; increased soil fertility and carbon storage; sequestration of carbon in the soil (Stagnari et al. 2017; Watson et al. 2017). Legume and cool-season grasses have similar concentrations of ADF, but grasses have higher NDF, which may limit DMI compared to legumes (Evers et al. 2011). A popular legume forage is alfalfa which can be either ensiled, as it is mainly preserved in the US, or dried as hay, most popular in Italy. It is a perennial crop that can grow for 3-4 years in an area with mild winters or 6-9 years with dormant periods during the winter season. The number of cuts (i.e. 2-12) depends on the climate (fao.org 2021). In the above-mentioned study of Gislon et al. (2020a), about 20% of the farm fed alfalfa silage, while more than 50% alfalfa hay (Figure 12), included mostly between 12 and 22% of diet DM. In Zucali (2018), 38% of the farm grew alfalfa (considering both silage and hay as preservation method), with a yield of 11.5 t DM/ha; for hay the NDF and CP concentrations were 51.7% and 15.6%, respectively, while for silage 47.8% and 17.3%. Preservation as hay proved to increase the amount of ruminal bypass protein compared to silage, but digestibility in the solution of pepsin and HCl was lower (Hristov and Sandev 1998). Brito and Broderick (2006) tested different ratios of alfalfa and corn silage (from 51:0 to 10:40, expressed as inclusion on total DM): the higher CP concentration of alfalfa allowed to reduce soybean meal inclusion in the diet, from 16.1% of the diet with 10% alfalfa silage to 2.95% of the diet with 51% alfalfa silage. The authors found that DMI and milk yield decreased linearly when alfalfa was replaced by corn silage. However, up to the ratio alfalfa silage:corn silage of 37:13, DMI was only numerically decreased compared to the treatment with the highest inclusion of alfalfa silage; instead, milk yield was not decreased up to inclusion of alfalfa silage almost equal to corn silage (ratio 24:27). Milk protein concentration was higher with higher inclusion of corn silage, but not different from the treatments with ratio 24:27 and 37:13 between the two silages; protein yield was not affected by the diet.

Soybean silage. Soybean is another legume species. Growing on the farm this crop and preserving it as whole-plant silage could represent an alternative, high-protein forage since Tabacco et al. (2018a) found a yield of DM for soybean silage equal to 5.4 t/ha, corresponding to 1.04 t/ha of CP, in Piedmont region (North Italy). Besides the positive agronomical effects mentioned above for alfalfa, soybean has the advantage of being an annual crop. Thus, it can be inserted in rotation with corn with higher flexibility because the land for crop production is not occupied over multiple years. Furthermore, farmers have more opportunities to apply manure, and they can use the same equipment applied for corn cropping (Seiter et al. 2004). Soybean has a wide window where it could be harvested for silage production, but between reproductive stage 6 (R6) and 7 (R7) (Fehr and Caviness 1977), it provides the highest quality since nutrients accumulate in the pods, increasing the concentration of CP and decreasing the ones of ADF and NDF (Seiter et al. 2004). Spanghero et al. (2015) wilted soybean prior to ensiling to have higher DM concentration in the silage. They investigated three different maturity stages at harvest (R4, R5, and R6), finding increasing values of NDFD (from 31.9 to 46.5%) and CPD (from 39.1 to 54.8%) with advancing maturity stage. Despite high buffering capacity (18.1, 9.4% of protein and ash, respectively), low starch content (3.5%), and low DM concentration at harvesting (26.4%), soybean proved to be adequately preserved as silage (i.e. rapid decline in pH and sharp increase in lactic acid concentration) (Mustafa and Seguin 2003).

Rigueira et al. (2015) improved the quality of soybean silage, preserving it with the addition of molasses and a microbial inoculant: in this way, digestibilities of DM, CP, NDF, and NFC were improved in beef cattle. The cultivar used as well can affect the quality of the silage. Generally, the forage cultivars are characterized by greater vegetative growth than the grain cultivars, and thus they are taller, have higher leaf and stem DM, and have higher DM yield (Darmosarkoro et al. 2001). Tabacco et al. (2018b) found that low size plants had higher NDFD compared to medium-size ones (50.6 vs. 46.6%), higher CP concentration (24.4 vs. 20.2%), and lower lignin concentration (6.6 vs. 9.1%) after 200 days of storage, when harvested at R7.

Only a few in vivo studies have been conducted feeding soybean silage to dairy cows to the best of our knowledge. Vargas-Bello-Pérez et al. (2008) run a study in Canada, comparing two diets with 36% of total DM coming either from soybean silage or alfalfa silage. DMI and milk yield were lower for the soybean silage diet, probably because of higher dietary NDF, but no effect was found on ECM and dairy efficiency. The treatment did not affect milk protein concentration even if higher milk urea nitrogen was found for cows fed soybean silage (15.6 v. 14.3 mg/dL of the alfalfa silage diet), probably for a lower concentration of non-structural carbohydrates. On the other hand, milk fat concentration was higher for the soybean silage diet (3.78 vs. 3.58%). Diet digestibility was not affected by the treatment. In one experiment conducted in Brazil (Ghizzi et al. 2020), four different inclusions were tested in partial replacement of corn silage: soybean silage represented the 0, 8, 16, or 24% of diet DM. The results were negative in terms of lactation performance since increasing inclusion levels linearly decreased DMI, milk yield, and milk components yield. Milk protein concentration linearly decreased as well, but the magnitude of the effect was negligible (from 3.29 to 3.25%). Digestibility was linearly decreased for DM (from 72.8 to 66.7%), OM (from 74.3 to 68.5%), NDF (from 57.2 to 47.3%), and CP (from 75.7 to 67.6%). The negative results concerning DMI were probably due to the higher intake of feed with long particle size and higher EE with soybean silage diets. In another study from Brazil, Silva et al. (2021) evaluated three diets: a control diet with 48% DM coming from corn silage and two diets where 8% of DM from corn silage was substituted with either black oat silage or soybean silage. Considering only the comparison between the corn silage and the soybean silage diets, DMI was reduced by soybean silage inclusion. On the contrary, no effect was found on nutrient digestibility, milk yield, milk quality, and dairy efficiency. Cows fed the soybean silage diet had lower N intake in g/d, but the diet did not affect milk and urinary N excretions and milk urea nitrogen. Comino et al. (2018) investigated the use of soybean silage in an Italian commercial dairy farm. The authors wanted to exploit this forage as a protein and fat source since this silage had 22.8 and 10.4% concentrations of CP and EE, respectively. For this reason, they formulated two diets, one with 2.7% soybean meal on total DM and 2% cotton seed, as protein and EE source; the other with 8.7% total DM from soybean silage in complete substitution of soybean meal and cotton seed. However, the main protein source in these two diets was canola meal, included as 7.6% of diet DM on average, and not soybean meal (contrary to Experiment 4 of the present PhD thesis). A decrease in milk yield was found but no effect on ECM due to higher protein and fat concentrations in the soybean silage diet. DMI was higher for the soybean silage diet while dairy efficiency for the control.

2. Aims of the study

The environmental impact of dairy cow nutrition related to enteric fermentation and LUC connected to soybean meal production was discussed above. As presented, several technical opportunities concerning dairy cow nutrition are available in order to mitigate these sources of impact. Thus, the overall aim of the present PhD project was to evaluate different dietary strategies directed at improving the dairy cows sector sustainability. The nutritional efficiency and cow productivity were also addressed, as this approach could decrease the environmental impact per unit of product. In particular, the addition of EO, as modulators of rumen fermentation, and a focus on the effect of the forage basis on CH_4 production were implied as strategies to mitigate enteric CH_4. Finally, a high-protein forage and a soybean meal not connected with LUC were evaluated as protein source alternatives to conventional soybean meal.

In chapter 3, the essential oil of *Achillea moschata* and its pure components were added to the substrates of an in vitro study. The effect of these additives was evaluated in terms of in vitro gas, CH_4, and VFA production. The effect on the microbial population was assessed considering the bacterial and protozoal community.

In chapter 4, the effect of different forage bases in the diet of dairy cows was evaluated through a meta-analysis approach. In vivo cow response to the main forage in the diet was analyzed considering CH_4 emission and lactation performance. Prediction models were proposed to estimate CH_4 production from quantitative factors related to diet characteristics and milk production.

In chapter 5, the IPCC (2019) Tier 2 model for predicting enteric CH_4 emission was evaluated using a dataset of Italian in vivo studies. An alternative model derived from this dataset was proposed and evaluated to predict better the CH_4 emitted by cows fed typical Italian diets.

In chapter 6, an in vivo experiment tested the inclusion of home-grown soybean silage in the diet of dairy cows. The whole-plant silage was used in partial replacement of imported soybean meal. The productive response of dairy cows was evaluated in terms of milk production, nutrient digestibility, and N balance.

In chapter 7, the use of soybean silage and responsible soy, compliant to FEFAC guidelines rather than conventional soybean meal, was evaluated in terms of environmental impact through an LCA approach. These solutions were evaluated considering the forages inserted in the ratio, the milk production, and the daily diet fed to the cows.

3. Experiment 1

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Evaluation of dietary addition of 2 essential oils from *Achillea moschata*, or their components (bornyl acetate, camphor, and eucalyptol) on in vitro ruminal fermentation and microbial community composition

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Abstract

This study investigated the effects of 2 *Achillea moschata* essential oils extracted from plants collected in 2 different valleys of the Italian Alps and 3 pure compounds of oils — bornyl acetate (BOR), camphor (CAM), and eucalyptol (EUCA) — on in vitro ruminal fermentation and microbiota. An in vitro batch fermentation experiment (Exp. 1) tested the addition of all of the substances (2 essential oils and 3 compounds) in fermentation bottles (120 mL) at 48 h of incubation, whereas a subsequent in vitro continuous culture experiment (Exp. 2) evaluated the pure compounds added to the fermenters (2 L) for a longer incubation period (9 d). In both experiments, total mixed rations were incubated with the additives, and samples without additives were included as the control (CTR). Each treatment was tested in duplicate and was repeated in 3 and 2 fermentation runs in Exp. 1 and 2, respectively. Gas production (GP) in Exp. 1 was similar for all of the treatments, and short chain volatile fatty acid (SCFA) production was similar in both experiments except for a decrease of SCFA produced ($P = 0.029$) due to EUCA addition in Exp. 2. Compared to CTR, BOR and CAM reduced the valerate proportion ($P = 0.04$) in Exp. 1, and increased ($P < 0.01$) the acetate proportion in Exp. 2. All treatments increased ($P < 0.01$) total protozoa counts (+ 36.7% and + 48.4% compared to CTR on average for Exp. 1 and 2, respectively). In Exp. 1, all of the treatments lowered the Bacteroidetes and Firmicutes and increased the Proteobacteria relative abundances ($P < 0.05$), whereas in Exp. 2, the EUCA addition increased ($P = 0.012$) the *Ruminococcus*. In Exp. 1, methane (CH_4) as a proportion of the GP was lowered ($P = 0.004$) by the addition of CAM and EUCA compared to CTR, whereas in Exp. 2, EUCA reduced the amount of stoichiometrically calculated CH_4 compared to CTR. Overall, essential oils extracted from *A. moschata* and the pure compounds did not depress in vitro rumen fermentation, except for EUCA in Exp. 2. In both experiments, an increase of the protozoal population occurred for all the additives.

Keywords

Methane; Rumen fermentation; Achillea moschata; Protozoa; Microbiome

1. Introduction

Methane (CH_4) is a greenhouse gas produced by ruminal microorganisms as a consequence of enteric fermentation. Although CH_4 is an inevitable product of fermentation, its emission can be decreased by supplementing ruminant diets with specific additives and ingredients. Plants produce a wide variety of secondary metabolites to prevent disease, pest, and predator attacks (Jouany and Morgavi, 2007). Among secondary metabolites, essential oils have been widely evaluated as feed additives for improving microbial metabolism in the rumen and inhibiting methanogenesis (Calsamiglia et al., 2007; Patra and Yu, 2012; Pirondini et al., 2015). Essential oils are obtained from the steam distillation of plants and include a variable mixture of different compounds, like terpenes and phenylpropanoid derivatives (Aziz et al., 2018), characterized by different properties (Bakkali et al*.,* 2008). Many of the active compounds in essential oils have antimicrobial activity. Antimicrobial activity should be evaluated for the selection of additives that can decrease CH_4 production without compromising overall fermentation in the rumen. Only a few individual active compounds of essential oils have been tested for their effects on rumen fermentation and CH_4 production (Joch et al., 2018). In recent studies (Joch et al., 2016; Joch et al., 2018), compounds such as bornyl acetate (BOR) and camphor (CAM) decreased CH_4 production without adverse effects on short chain fatty acid (SCFA) production during short-term in vitro incubations. Together with eucalyptol (EUCA), CAM and BOR represent the main components of *Achillea moschata* essential oil (Vitalini et al., 2016). *Achillea moschata* is a plant that has antimicrobial properties (Vitalini et al., 2016), and which has traditionally been used to treat human digestive disorders as well as animal ones (Vitalini et al., 2015). The antibacterial properties of the essential oil from *Achillea* spp. are likely to be due to its high concentration of CAM (about 27% of the oil) and EUCA (about 11% of the oil) (Si et al., 2006). Furthermore, in recent studies (Fidan et al., 2019; Zerkani et al., 2019; Baali et al., 2019), essential oils having BOR among their main constituents also showed antimicrobial activity.

Another issue in the search for feed additive-based mitigation strategies is that the rumen's microbial ecosystem may adapt to the inclusion of feed additives to the diet. In that case, only a transient reduction of CH_4 emissions may be achieved (Klop, 2016). Cardozo et al. (2004) reported a transient effect of plant extracts on fermentation characteristics that disappeared after 6 d, indicating that microbial adaptation can occur after short-term exposure. Hence, the evaluation of the additive by a continuous rumen fermenter is another step that should be performed before conducting an in vivo study. This study hypothesized that the use of *A. moschata* essential oils and their main pure compounds could affect rumen fermentation patterns because of a change in the rumen microbiome communities resulting in lower CH_4 production without negatively affecting fermentability. The objective of the current study was to investigate the effect of *A. moschata* essential oils collected from 2 different locations in Italy and their main pure compounds (BOR, CAM, and EUCA) on the rumen fermentation and the microbiota. In vitro batch fermentation systems were used to screen the essential oils and pure compounds. In contrast, a continuous culture system was used to evaluate the potential adaptation of microbiota to the oils' pure compounds.

2. Materials and Methods

The study consisted of 2 in vitro rumen fermentation experiments: a batch fermentation at the Department of Agricultural and Environmental Sciences in Milano (Exp. 1) and a continuous culture fermentation at the Department of Agricultural, Food, Environmental, and Animal Sciences in Udine (Exp. 2). Experiment 1 used fistulated animals, which were handled as outlined by the Directive 2010/63/EU on animal welfare for experimental animals, according to the University of Milan Welfare Organism (OPBA) and with authorization number 904/2016-PR from the Italian Ministry of Health.

2.1 Plant material, essential oils, and pure compounds

Achillea moschata was collected during the blossom period in July 2017 in 2 different (in terms of altitude, geomorphology, lithology, and temperature influencing the essential oil composition) valleys of the Rhaetian Alps (located in Sondrio Province, Northern Italy). Specifically, the samples were harvested at 2,400 m in Valfurva and 2,000 m in Valchiavenna. Two voucher specimens (No. AMVF 104 and No. AMVC105, respectively) were deposited at the Department of Agricultural and Environmental Sciences, Milan State University (Milan, Italy) after their identification, according to the morphological traits described in Flora d'Italia (Pignatti, 1982). The air-dried aerial parts (50 g) of *A. moschata* were subjected to steam distillation for 1 h in a Clevenger-type apparatus. The obtained distillates were dried over anhydrous sodium sulfate and were concentrated with a rotary evaporator at 30 °C to produce pale blue yellow oils. The 3 main compounds of *A. moschata* essential oil, BOR, CAM, and EUCA, were purchased from Sigma-Aldrich (Milan, Italy).

2.2 Experiment 1: in vitro batch fermentation

Ruminal fluid was collected from 2 fistulated dry Italian-Friesian dairy cows that were fed a total mixed ration (TMR) composed of corn silage, grass hay, cornmeal, and soybean meal (434, 323, 105, and 136 g/kg DM, respectively); the diet chemical analysis was CP 145, NDF 420, NFC 330, and ash 650 g/kg DM. The cows were fed the TMR twice daily (07:00 and 19:00) to achieve DM intake of 8 kg/d.

Rumen liquor was collected 2 h after the morning feeding. The liquor was strained through 4 layers of cheesecloth and poured into a flask, pre-warmed at 39 °C, and purged with carbon dioxide (CO_2). The buffer solution was prepared according to Menke and Steingass (1988). The fermentation substrates were 2 TMR and the main ingredients were corn silage, cornmeal, solvent extracted soybean meal, alfalfa and grass hays (352, 172, 170, 145 and 133 g/kg DM on average, respectively) and contained 155 and 348 g/kg DM of CP and NDF, respectively. Approximately 200 mg of each TMR was weighed in duplicate in serum bottles (120 mL), and each bottle was inoculated with 30 mL of rumen inoculum and the experimental additives (2 *A. moschata* essential oils, BOR, CAM, and EUCA), following the procedure of Menke and Steingass (1988). A control sample (CTR; i.e., sample without additive) was also incubated. Each compound was dissolved in ethanol, and the compound concentration used was at 200 mg/L of inoculum. The concentration of ethanol in the final inoculum was 0.67% (vol/vol), in agreement with the findings of Benchaar et al. (2007a), where the final concentration of ethanol in culture fluid was less than 2% (vol/vol). The same amount of ethanol was added to CTR.

For each additive (2 *A. moschata* essential oils, BOR, CAM, and EUCA), a corresponding blank (inoculum + ethanol + additive) was incubated, and each additive was tested against the CTR (TMR + inoculum + ethanol).

Three incubation runs were conducted in a shaking water bath at 39 °C for 48 h. At 24 and 48 h of incubation, headspace pressure was recorded using a digital manometer (model 840082, Sper Scientific, Scottsdale, AZ, USA), and a sample of air was collected from the bottle headspace using a gas-tight syringe (Hamilton, USA) and stored in gas-tight vials (Labco Exetainer Vials, UK). The gas pressure data recorded at each time-point were converted to the volume of gas produced (GP) using the ideal gas law. At the end of the incubation, pH was recorded, and 3 samples of liquor were collected: 10 mL for rumen microbiota characterization, 5 mL for the protozoa count, and 5 mL for SCFA determination. For protozoa analysis, 5 mL of 50% formalin solution was added to the samples, whereas for SCFA analysis the samples were acidified with 5 mL of 25% metaphosphoric acid. The samples for microbiota and SCFA were immediately frozen.

Gas samples were analyzed for CH_4 concentration using an Agilent 3000A GC gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a thermal conductivity detector (170 °C) using a stainless steel column (Carboxen 1000, 60/80 mesh Supelco, USA) with helium as the carrier gas (30 ml/min, isothermal oven temperature: 120 °C). Gas calibration was completed using a standard mixture of CO_2 and CH_4 (SAPIO, Italy) with 4 points of calibration. The SCFA concentrations were determined using a Varian 3800 gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA), following the guidelines of Pirondini et al. (2012).

2.3 Experiment 2: in vitro continuous fermentation

Eight 2000 mL single-flow continuous fermenters (1,500 mL of effective volume) were used, as described in Mason et al. (2015), to perform 2 fermentation runs, which lasted 9 d each with 6 d of adaptation and 3 d of sampling. In each fermentation, the rumen fluid was collected in the same slaughterhouse from 4 culled dairy cows, which were fed a TMR based on corn silage, grass hay, cornmeal, and soybean meal and were slaughtered in healthy conditions. The rumen fluid was collected immediately after the slaughter of cows (in the morning, after about 12 h from the last feeding of animals) and transported to the laboratory within half an hour in airtight glass bottles, refluxed with CO_2 and maintained at 39 °C. The fermentation substrate was a TMR composed of corn silage, cornmeal, hay, soybean meal and a micromineral and vitamin premix (370, 260, 180, 170 and 20 g/kg DM, respectively), which was dried at 60 °C (48 h) and then coarsely ground; it contained 150 and 350 g/kg of CP and NDF on DM, respectively. The treatments consisted of the daily addition of the 3 pure compounds—BOR, CAM, and EUCA—to the fermentation fluid (100 mg/L) of the fermenters (2 fermenters per treatment within each fermentation run) as well as using 2 fermenters with only ethanol (CTR) added. The pure compounds and the standard diet were provided to each fermenter twice a day in equal doses (at 09:00 and 17:00) for a total of 18 g/d of DM. Artificial saliva (Slyter et al., 1966) was continuously infused using a peristaltic pump at 1.3 mL/min. During the last 3 d before morning feeding, the pH was directly measured (GLP 22, Crison Instruments, S.A. Barcelona, Spain), whereas samples for the ammonia-nitrogen, SCFA, protozoa and bacterial DNA (10, 5, 5, and 1 mL sample, respectively) were collected for later analysis. The samples for the ammonia-nitrogen were stored at -20 °C, thawed at room temperature, and then analyzed using an ammonia electrode (Ammonia Gas Sensing Combination Electrode, Hach Company, 2001). Samples for SCFA analysis were mixed with 5 mL of 0.05 mol/L H_2SO_4 and were stored at -20 °C; after thawing, they were centrifuged at 20,000 × *g* for 30 min at 20 °C and filtered by a polypore filter (0.45 µm, Agilent Technologies, Milan, Italy).

The SCFA concentration was measured as described by Martillotti and Puppo (1985).

The CH_4 yield was estimated by the equation of Moss et al. (2000), considering a hydrogen recovery of 90% (default):

$$CH_4\ (\text{mmol/L}) = 0.45\ (\text{acetic, mmol/L}) - 0.275\ (\text{propionic, mmol/L}) + 0.40\ (\text{butyric, mmol/L})$$

2.4 Protozoa and Microbiome Analysis

Protozoa were counted as described by Dehority (2003). For the extraction of the DNA from the rumen microbiota, 350 µL of rumen fluid was stored at -80 °C pending extraction. Particular attention was devoted to this operation; 350 µL were taken immediately after shaking, as the rumen fluid has rapid precipitation. In this way, all of the analyzed samples had the same characteristics. The DNA from the rumen fluid was extracted using the NucleoSpin Soil kit (Macherey-Nagel, Germany) following the procedures and using the reagents suggested by the kit manufacturer.

2.5 PCR amplification of 16S gene, PCR products sequencing, and bioinformatics analyses

In order to identify the bacterial community present in the rumen fluid, a portion of the 16S gene was used, as described by Takahashi et al. (2014). For the amplification, the following primers were used: Pro341F: 5’-CCTACGGGNBGCASCAG -3’ and Pro805R: Rev 5’-GACTACNVGGGTATCTAATCC -3. The amplifications were performed using 5 µL of the extracted DNA in a final reaction volume of 25 µL using Platinum Taq DNA polymerase high fidelity (Thermofisher, MA, USA), following the manufacturer instructions. The amplifications were performed for 27 cycles using 55 °C as the annealing temperature. The libraries were purified with Beads Amplure XP 0.8X, amplified with Indexes Nextera XT Illumina; they were normalized, mixed, and loaded on Miseq using the 2x300bp (paired-end) approach to generate a minimum of 50,000 sequences (± 20%). The raw sequences R1 and R2 (raw reads) were verified and filtered by quality, trimmed by the primers, and fused by Qiime2 v8 software. DADA2 (Qiime2) software isolated the ASVs (formerly OTUs), whose sequences were compared against the Greengenes v13-8 to obtain the taxonomic assignment.

2.6 Statistical Analyses

The data from Exp. 1 and 2 were statistically analyzed by the proc mixed procedure of SAS 9.4 (SAS Institute Inc., Cary, NC USA), with the following model: $Y_{ijk} = \mu + \alpha_i + \beta_j + \varepsilon_{ijk}$, where Y_{ijk}, is the dependent variable; µ is the overall mean; α_i is the random effect of the fermentation run (i = 1 to 3 in Exp. 1 and i = 1 to 2 in Exp. 2); β_j is the fixed effect of the dietary treatment (j = 1 to 6 in Exp.1 and j = 1 to 4 in Exp. 2); and ε_{ijk} is the random error. The least square means were reported. For all of the statistical analyses, significance was declared at $P \leq 0.05$ and trends at $P \leq 0.10$.

The linear regression analysis between CH_4 production (% of the GP total) and the main bacterial phyla and protozoa was performed by the proc reg procedure of SAS 9.4. Correlation analysis between the main bacterial phyla and protozoa was performed using the Pearson correlation method and the proc corr procedure of SAS 9.4.

3. Results

3.1 Experiment 1. Gas and CH_4 Productions, and Rumen Fermentation Parameters

The results of the GP are shown in Table 1. The GP (mL/200 mg DM) was not affected by the additive. The CH_4 production as a percentage in the total GP at 48 h was lower for EUCA (22.5% in total GP) and CAM (22.4% in total GP), as compared to the CTR (24.2% in total GP) ($P = 0.044$). Among the other parameters, tendencies ($P < 0.10$) were found at 24 and 48 h for CH_4 production (mL/200 mg DM) and for the CH_4 percentage in the total GP at 24 h, with EUCA and CAM being the most promising compounds. The 2 essential oils did not affect any of the parameters evaluated.

The pH and total SCFA were not affected by the treatments (Table 2). The CTR had the highest values (% of the SCFA) for *iso*-butyrate (1.74) ($P = 0.003$) and for *iso*-valerate (3.11) ($P < 0.001$), as compared to the other treatments (on average 1.62 and 2.79, respectively, for *iso*-butyrate and *iso*-valerate). Bornyl acetate and CAM reduced valerate (on average 1.69, % SCFA) as compared to CTR (1.81) ($P = 0.04$). The acetate to propionate ratio was not affected by the treatment ($P = 0.299$).

3.2 Protozoa count and relative abundance of the main bacterial phyla and Euryarchaeota

The results of the protozoa count and the relative abundances of the main bacterial phyla and Euryarchaeota are shown in Table 3. All of the compounds increased the total number of protozoa ($P = 0.006$) compared to the CTR. The genus *Entodinium* (% of the total protozoa) increased (on average 91.8 vs. 83.0, respectively, for samples treated with additives and CTR; $P < 0.001$), whereas all of the experimental treatments decreased ($P = 0.001$) the genus *Diplodinium* (13.6% vs. 6.6% of the total protozoa, for the CTR and treatments with additives, respectively). The percentage of the other protozoa (Ophryscolecinae*, Isotricha,* and *Dasytricha*) was lower ($P = 0.018$) in BOR, CAM, and EUCA (on average 0.91) compared to the CTR (3.38). There was a negative relationship ($P < 0.001$) between the total protozoa and CH_4 production (% in the total GP), although Ophryscolecinae were quadratically correlated ($P = 0.002$) to CH_4 production (Figure).

The additives affected the relative abundance of Bacteroidetes, Proteobacteria*,* and Firmicutes (P = 0.003, 0.014, and 0.014, respectively) as compared to CTR. Bacteroidetes and Firmicutes abundances decreased, whereas the Proteobacteria abundance increased for all treatments compared to CTR. No effect related to the additive was observed for the Euryarchaeota abundance ($P = 0.189$). The Euryarchaeota relative abundance was positively associated with CH_4 (% in total GP at 48 h) as follows:

Euryarchaeota relative abundance (%) = 0.0582 × CH_4 production (% in total GP) - 0.658; ($R^2 = 0.313$); (root mean square error [RMSE] = 0.146; $P = 0.059$). The correlations among the protozoa and the main bacterial phyla and Euryarchaeota are reported in Appendix Table. The most significant correlations showed that Euryarchaeota was positively correlated with Ophryscolecinae (r = 0.697; $P < 0.05$), but negatively correlated with *Entodinium* (r = -0.584; $P < 0.05$). In contrast, Proteobacteria was positively correlated with *Entodinium* (r = 0.658; $P < 0.05$). The regressions among the main bacterial phyla and the CH_4 emissions (% in total GP) are shown in Appendix Figure. Firmicutes and Bacteroidetes were positively related to CH_4 emission, whereas Proteobacteria and Spirochaetes were negatively correlated.

3.4 Exp. 2. continuous rumen fermenter system

The results of the continuous rumen fermenter are shown in Tables 4 and 5. The inclusion of BOR and EUCA increased the pH in the fermentation liquid ($P < 0.001$). The production of SCFA showed a decrease of about 20% when EUCA was added compared to CTR ($P = 0.029$), whereas this compound did not modify the proportions of different SCFA, except for an increase in the valerate ($P = 0.001$) in comparison with the other treatments. Bornyl acetate and CAM increased the proportion of acetate in the fermentation liquid by about 10% compared to the CTR (61.26%, 58.44%, and 54.34% for BOR, CAM, and CTR, respectively). Valerate was modified by BOR inclusion, resulting in a lower value ($P < 0.05$) than CTR and EUCA. The stoichiometrically calculated CH_4 was less for EUCA than for the other treatments (7.40 vs. 8.87 mmol/L on average, $P = 0.025$).

Protozoa were affected by the addition of the 3 compounds compared to CTR, with an increase of about 50% ($P < 0.001$). No modification for the protozoa groups was detected. The relative abundance of bacterial phyla and genera was affected by the addition of EUCA, which increased ($P = 0.011$) the presence of *Ruminococcus* (Table 5). Moreover, the addition of EUCA tended to increase Firmicutes and decrease Bacteriodetes ($P = 0.090$ and $P = 0.084$, respectively). The relative abundance of *Anaereovibrio* was lower ($P < 0.05$) for CAM and BOR compared to EUCA.

4 Discussion

Achillea moschata is a medicinal plant, which has been traditionally used in ethnomedicine to treat various digestive disorders in humans and animals. For this plant, Vitalini et al. (2016) showed a broad spectrum of antimicrobial activity against some food pathogen bacteria, such as *Bacillus cereus*, *Staphylococcus aereus*, *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. The present study aimed to evaluate the effects of the main pure compounds of *A. moschata* essential oil and the essential oil on in vitro rumen fermentation and the microbiome. To the best of our knowledge, the role of *A. moschata* essential oil in modifying rumen metabolism and enhancing nutrient utilization by animals has never been investigated. The main compounds of *A. moschata* essential oil are BOR, CAM, and EUCA. Interestingly, these compounds gave promising results for lower enteric CH_4 production in short-time in vitro incubations without decreasing SCFA production (Joch et al., 2016, 2018). In Exp. 1 (48 h of incubation), the GP and SCFA did not decrease with the additives, whereas in Exp. 2 (9 d of incubation), the addition of EUCA decreased SCFA production, which is possibly related to lower digestibility of the diet supplemented with EUCA. The difference between the 2 experiments might be due to several reasons, such as different doses used, the different donor animals, and the possible adaptation of rumen bacteria to the additives. The selected compounds were oxygenated monoterpenes, which degrade little in the rumen; for example, Malecky et al. (2012) showed that after 24 h of incubation with the caprine rumen inoculum, oxygen-containing terpenes were less degraded than linear and monocyclic terpenes. Hence, it can be speculated that in the short trial (Exp. 1), the additives were slowly degraded, whereas in Exp. 2, the rumen microbiome was probably more adapted to the additives. Similarly, Cardozo et al. (2004), using a continuous fermenter, observed a different SCFA profile due to essential oil supplementations at 2 d of fermentation, whereas these differences disappeared at longer fermentation lengths (e.g., 6 d) due to rumen microbial adaptation.

The SCFA profile was affected by the additive. In Exp. 2, EUCA increased the valerate proportion. Ungerfeld (2015) suggested that an increase in ruminal hydrogen availability, following methanogenesis inhibition, enhances the fermentation pathways that consume hydrogen, such as the formate, valerate, and caproate biosynthesis process. The estimated CH_4 production was lower for EUCA than for other treatments. On the other hand, CAM and BOR increased the acetate proportion (without affecting the acetate to propionate ratio) compared to CTR, which was likely related to a better fiber digestibility. According to this hypothesis, the pH value of BOR was higher than that of CTR.

In both experiments, the 3 dominant bacterial phyla were Bacteroidetes, Firmicutes, and Proteobacteria, but with a different ratio in the CTR samples (i.e., ethanol) between the 2 experiments (e.g., 31:37:23 in Exp.1 and 55:27:11 in Exp. 2, for Bacteroidetes, Firmicutes, and Proteobacteria, respectively). Other studies (Jami et al., 2014; Li et al., 2009) reported a considerable variation between animals concerning the abundance of the main bacterial phyla. In the present study, the rumen inoculum for the 2 experiments was collected from different animals fed different diets and reared in different conditions, which probably caused the observed difference.

In Exp. 1 (48 h), all of the treatments increased the relative abundance of Proteobacteria and decreased that of Firmicutes and Bacteroidetes. Wallace et al. (2015) found out that in beef cattle, there was a 4-fold abundance of Proteobacteria in animals with lower CH_4 emissions compared to those with higher emissions. Similarly, Danielsson et al. (2017) reported a higher abundance of Proteobacteria in low-CH_4 emitting cows than in high-CH_4 emitting ones. These results appear to agree with the results for the EUCA and CAM treatments in Exp. 1.

Although the effects were less marked, in Exp. 2, there was a higher Firmicutes-to-Bacteroidetes ratio for EUCA than for CTR, and the Firmicutes-to-Bacteroidetes ratio was found to be strongly correlated with daily milk-fat yield (Jami et al. 2014). However, Delgado et al. (2019) found that more efficient cows presented a higher relative abundance of Bacteroidetes and a lower, but not significant, relative abundance of Firmicutes. A lower amount of Bacteroidetes in the digestive microbiota was also associated with an impaired feed conversion rate, residual feed intake (Jami et al. 2014), and an increased fat deposition in mice (Turnbaugh et al. 2006). Overall, a lower amount of Bacteroidetes in the rumen might redirect energy intake, resulting in an increased fat deposition at the expense of lowering milk production per unit of feed intake (Delgado et al. 2019). Hence, the possible use of EUCA as a feed additive should be carefully evaluated. Another effect observed in Exp. 2, due to the EUCA treatment, was the increase in the relative abundance of *Ruminococci*. The bacteria belonging to this genus played a fundamental role in cellulose degradation (Christopherson et al., 2014). However, the increase of the abundance of *Ruminococci* was not associated with a change in the fermentative pattern (i.e., acetate proportion); on the contrary, a decrease in the SCFA concentration was observed with EUCA supplementation.

The additives had no effect on the relative abundance of Euryarchaeota in the present study. Shi et al. (2014) suggested that the number of Euryarchaeota was not as important for the CH_4 yield as the metabolic activity of individual methanogenic species. However, in the present study, there was a positive correlation between Euryarchaeota and CH_4 production, expressed as a percentage of the total gas.

Supplementation with the additives markedly increased the total number of protozoa (i.e., 30-50%) in both experiments, despite the different experimental conditions (e.g., inoculum donor animals and length of incubation and doses). In agreement with these results, a study from Broudiscou et al. (2000) showed that *A. millefolium* increased large ruminal protozoa. The effect of essential oils on the protozoal population varies in the literature. Some studies reported a lack of effect (Benchaar et al., 2007b; Newbold et al., 2004), whereas others found that essential oils had a stimulatory effect on the protozoa (Patra et al. 2006), although the mechanism was not elucidated (Patra and Saxena, 2010). An opposite trend between the in vitro CH_4 yields and the protozoa counts was found in our experiments, which was unexpected given the assumed role protozoa plays in the rumen methanogenesis process (Newbold et al., 2015). A meta-analysis by Guyader et al. (2014) concluded that methanogenesis is also regulated by other mechanisms besides protozoa numbers because, in several experiments, a variation in CH_4 emission was observed without corresponding changes in the protozoa numbers. In addition, Sarnataro et al. (2020) changed the concentration of the in vitro protozoa population by adding secondary plant compounds; however, the authors did not measure a variation in the CH_4 yield. Similarly, Wenner at al. (2020), in a continuous culture experiment, found that CH_4 yield was not decreased by defaunation. Different protozoa may have differing effects on rumen CH_4 production. Belanche et al. (2014) demonstrated that holotrich protozoa have a different endosymbiotic correlation with methanogens than entodiniomorphids. These differences may explain the more significant impact of holotrich protozoa on rumen methanogenesis than the entodiniomorphids (Belanche et al. 2015). The results of Exp. 1 demonstrated a quadratic relationship between CH_4 and Ophryscolecinae, which were also negatively correlated to Proteobacteria. To the best of our knowledge, this relationship has never been reported in the literature.

The role of protozoa in the rumen metabolism is not yet well defined, but some concerns arise from their contribution to CH_4 and ammonia yields in the rumen. However, protozoa are a relevant fraction of the natural microbiota of the rumen and have some beneficial effects on the nutrition of the host. Protozoa engulf starch granules and are, therefore, considered able to attenuate the risks of rumen acidosis. Moreover, protozoa may contribute to fiber digestion. Newbold et al. (2015) showed that the elimination of rumen protozoa significantly decreased NDF (–20%) and ADF digestibility (–16%), probably as a result of the loss of protozoal fibrolytic activity. Recent investigations also suggested possible positive effects related to protein nutrition because protozoa contributed 10 to 30% of the duodenal microbial protein flow (Sylvester et al., 2005) and were characterized by a higher lysine content than the rumen bacteria (Sok et al., 2017). Finally, the greater proportion of unsaturated fatty acids and CLA in the protozoa organisms could increase the supply of beneficial fatty acids for lower gut absorption by the ruminants (Or-Rashid et al., 2011). Given these considerations, a dietary additive that can increase rumen protozoa may have interesting applications that should be further evaluated.

4. Conclusions

The results of the present study indicated that the essential oils from *A. moschata* did not depress in vitro rumen fermentation, except for EUCA, and may have some interesting effects on rumen microbiota. Some of the compounds (EUCA and CAM) were able to reduce in vitro CH_4 yield, but this effect was not associated with the evident modifications of the methanogenic bacteria. In addition, there was a clear and relevant stimulation of all the essential oils studied in the protozoal population, which was also maintained during long-lasting incubations without adaptive phenomena. The lack of correspondence between the total protozoa counts and the CH_4 yield in the in vitro conditions requires further research efforts to elucidate the relationship between methanogens and protozoa better.

Author contributions

Stefania COLOMBINI: writing original draft, review and editing, conceptualization, data elaboration, funding acquisition; Andrea ROTA GRAZIOSI: 48 h in vitro analysis, writing review and editing, data elaboration; Pietro PARMA: microbiome analysis, data elaboration; Marcello IRITI: selection and proposal of *A. Moschata* plant material, oil extraction and processing, writing review and editing; Sara VITALINI: selection of plant material and oil extraction and processing, writing review and editing; Chiara SARNATARO: continuous fermenter analysis, writing review and editing, data elaboration. Mauro SPANGHERO: conceptualization, data elaboration, writing review and editing, funding acquisition.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that could inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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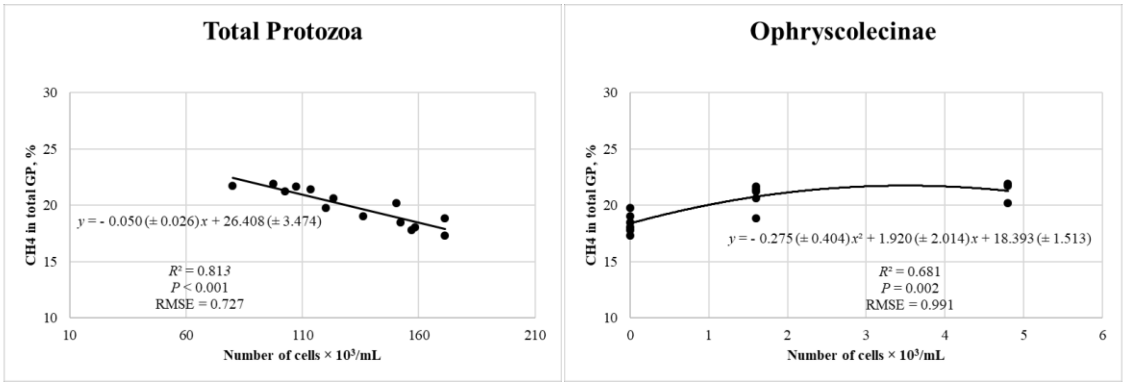


Figure. Linear and quadratic regressions between total protozoa count and Ophryscolecinae and CH_4 emission (% total GP 48 h). GP = gas production; RMSE = root mean square error.

Tables

Table 1. Gas production (GP) and CH_4 production of diets with the different additives in the Exp.1 (mL/200 mg DM unless otherwise stated).

Item		Pure compound[2]			Essential oil[3]		SEM	*P*-value
	CTR[1]	BOR	CAM	EUCA	OIL 1	OIL 2		
GP 24 h	31.1	30.2	30.3	30.3	30.5	30.0	2.90	0.772
GP 48 h	46.3	45.2	44.7	44.7	45.2	44.8	2.97	0.545
GP 24–48 h	15.2	15.0	14.4	14.4	14.6	14.8	1.05	0.334
CH_4 24 h	7.84	7.44	7.02	7.06	7.97	7.52	1.18	0.063
CH_4 48 h	11.3	10.9	10.2	10.2	11.1	10.5	1.54	0.075
CH_4 24–48 h	3.59	3.68	3.35	3.35	3.32	3.31	0.747	0.744
CH_4 24 h, %	22.9	22.1	20.9	21.0	23.6	22.5	1.03	0.082
CH_4 48 h, %	24.2^{a}	23.8^{ab}	22.4^{b}	22.5^{b}	24.4^{a}	23.2^{ab}	2.06	0.044

[1] CTR, control.

[2] BOR, bornyl acetate; CAM, camphor; EUCA, eucalyptol.

[3] OIL 1, *Achillea moschata* essential oil derived from sample 1; OIL 2, *Achillea moschata* essential oil derived from sample 2.

[a, b] Means in the same row with different superscripts are statistically different at $P < 0.05$.

Table 2. Rumen fermentation parameters of diets with the experimental additives in the Exp. 1

Item		Pure compound[2]			Essential oil[3]		SEM	*P*-value
	CTR[1]	BOR	CAM	EUCA	OIL 1	OIL 2		
pH	6.74	6.68	6.70	6.73	6.72	6.77	0.096	0.207
SCFA, mmol	63.8	62.4	60.6	59.3	59.2	57.7	5.29	0.189
SCFA percentage								
Acetate (A)	63.1	64.5	64.2	63.3	63.5	63.4	1.61	0.286
Propionate (P)	17.2	16.7	17.0	17.3	17.2	17.3	0.870	0.594
iso-butyrate	1.74^{a}	1.58^{c}	1.59^{bc}	1.65^{b}	1.65^{b}	1.64^{bc}	0.060	0.003
Butyrate	13.0	12.7	12.8	13.1	13.1	13.0	0.480	0.481
iso-valerate	3.11^{a}	2.83^{b}	2.72^{b}	2.79^{b}	2.83^{b}	2.80^{b}	0.045	<0.001
Valerate	1.81^{a}	1.69^{b}	1.69^{b}	1.75^{ab}	1.80^{a}	1.78^{ab}	0.187	0.040
A:P	3.68	3.89	3.81	3.67	3.70	3.68	0.287	0.299

[1] CTR, control.

[2] BOR, bornyl acetate; CAM, camphor; EUCA, eucalyptol.

[3] OIL 1, *Achillea moschata* essential oil derived from sample 1; OIL 2, *Achillea moschata* essential oil derived from sample 2.

[a, b, c] Means in the same row with different superscripts are statistically different at $P < 0.05$.

Table 3. Rumen protozoa count and relative abundance of main bacterial phyla in the Exp. 1

Item		Pure compound[2]			Essential oil[3]		SEM	*P*-value
	CTR[1]	BOR	CAM	EUCA	OIL 1	OIL 2		
Total protozoa, × 10^3 cell/mL	68.0^b	92.0^a	104.0^a	92.3^a	89.5^a	86.9^a	26.3	0.006
Total protozoa, %								
Entodinium	83.0^b	93.6^a	92.4^a	93.8^a	89.8^a	89.4^a	1.81	<0.001
Diplodinium	13.6^a	5.85^b	6.69^b	4.92^b	7.30^b	8.24^b	1.31	0.001
Other protozoa	3.38^a	0.577^c	0.900^c	1.24^{bc}	2.88^{ab}	2.35^{ab}	0.953	0.018
Phyla, % (total observations)								
Firmicutes	30.7^a	25.8^b	27.8^b	25.1^b	26.9^b	26.0^b	0.802	0.031
Bacteroidetes	37.1^a	32.8^b	27.7^d	28.3^{cd}	30.9^{bcd}	31.8^{bc}	1.11	0.014
Proteobacteria	22.5^b	32.8^a	36.7^a	39.7^a	36.5^a	34.4^a	1.95	0.014
Spirochaetes	3.59	4.67	4.61	3.77	2.98	4.52	0.375	0.107
Euryarchaeota	0.71	0.39	0.41	0.45	0.46	0.51	0.077	0.189
Others	5.31	3.52	2.82	2.71	2.35	2.80	1.01	0.445

[1] CTR, control.

[2] BOR, bornyl acetate; CAM, camphor; EUCA, eucalyptol.

[3] OIL 1, *Achillea moschata* essential oil derived from sample 1; OIL 2, *Achillea moschata* essential oil derived from sample 2.

[a, b, c, d] Means in the same row with different superscripts are statistically different at $P < 0.05$.

Table 4. Rumen fermentation parameters and protozoa count in the Exp. 2

Item		Pure compound[2]				
	CTR[1]	BOR	CAM	EUCA	SEM	*P*-value
pH	5.98^{b}	6.15^{a}	6.06^{b}	6.20^{a}	0.032	<0.001
Ammonia, mg/dL	13.3	13.9	13.9	12.6	0.543	0.356
SCFA, mmol	33.3^{a}	31.0^{ab}	32.3^{a}	26.8^{b}	1.36	0.029
SCFA, % (total SCFA)						
Acetate	54.3^{b}	61.3^{a}	58.4^{a}	51.5^{b}	1.29	0.001
Propionate	15.4^{ab}	16.0^{ab}	17.3^{a}	12.5^{b}	1.04	0.048
iso-butyrate	0.441	0.255	0.312	0.159	0.071	0.092
Butyrate	17.2	13.8	13.9	19.8	1.55	0.055
iso-valerate	0.917	0.802	0.917	0.905	0.110	0.853
Valerate	11.7^{b}	7.99^{c}	9.21^{bc}	15.2^{a}	0.874	0.001
A:P	3.55	4.04	3.42	4.15	0.285	0.250
Calculated CH_4[3] mmol/L	9.02^{a}	8.75^{a}	8.85^{a}	7.40^{b}	0.344	0.245
Total protozoa, × 10^3 cell/mL	54.9^{b}	84.1^{a}	80.7^{a}	79.6^{a}	2.76	<0.001
total protozoa, %						
Entodinium	95.3	95.5	95.1	95.4	0.911	0.990
Diplodinium	3.52	3.10	3.93	3.08	0.883	0.889
Other protozoa	1.20	1.38	1.00	1.52	0.599	0.934

[1] CTR, control.

[2] BOR, bornyl acetate; CAM, camphor; EUCA, eucalyptol.

[3] CH_4 was calculated according to the equation described by Moss et al (2000).

[a, b, c] Means in the same row with different superscripts are statistically different at $P < 0.05$.

Table 5. Relative abundance of main bacterial phyla and genera in the Exp. 2

Item		Pure compound[2]			
	CTR[1]	BOR	CAM	EUCA	SEM	*P*-value
OTU	404	323	398	351	65.4	0.788
Phyla, % (total observations)						
Firmicutes	55.1	54.2	54.8	62.8	2.43	0.090
Bacteroidetes	26.6	30.8	31.0	22.1	2.48	0.084
Proteobacteria	10.8	9.28	7.83	6.48	1.11	0.088
Spirochaetes	2.93	1.98	2.18	3.05	0.410	0.224
Actinobacteria	2.70	2.43	2.65	2.78	0.381	0.923
Euryarchaeota	0.985	0.680	0.743	0.885	0.222	0.765
Others / Unclassified	1.93^{ab}	1.33^{b}	1.60^{ab}	2.85^{a}	0.251	0.040
Genera, % (total observations)						
Anaeroplasma	0.110	0.162	0.310	0.143	0.064	0.113
Anaerovibrio	0.520^{ab}	0.333^{b}	0.290^{b}	0.575^{a}	0.737	0.028
Bifidobacterium	2.08	1.60	2.19	2.08	0.377	0.675
Butyrivibrio	4.87	3.39	5.93	4.53	0.714	0.118
Clostridium	0.885	0.489	1.09	1.44	0.423	0.419
Fibrobacter	0.288	1.34	0.043	0.233	0.555	0.361
Megasphaera	7.21	5.55	8.55	6.09	1.36	0.366
Methanobrevibacter	0.585	0.740	0.550	0.830	0.264	0.707
Oscillospira	1.28	0.880	1.61	2.29	0.617	0.378
Prevotella	20.2	15.7	22.4	15.8	3.79	0.441
Pseudobutyrivibrio	0.758	1.15	1.98	1.93	0.521	0.330
Ruminococcus	1.18^{b}	0.813^{b}	1.37^{b}	2.60^{a}	3.72	0.011
Shuttleworthia	5.11	3.52	3.32	9.19	2.33	0.214
Succiniclasticum	4.10	4.70	5.03	4.67	1.19	0.993
Succinivibrio	0.623	1.95	2.37	1.17	0.972	0.496

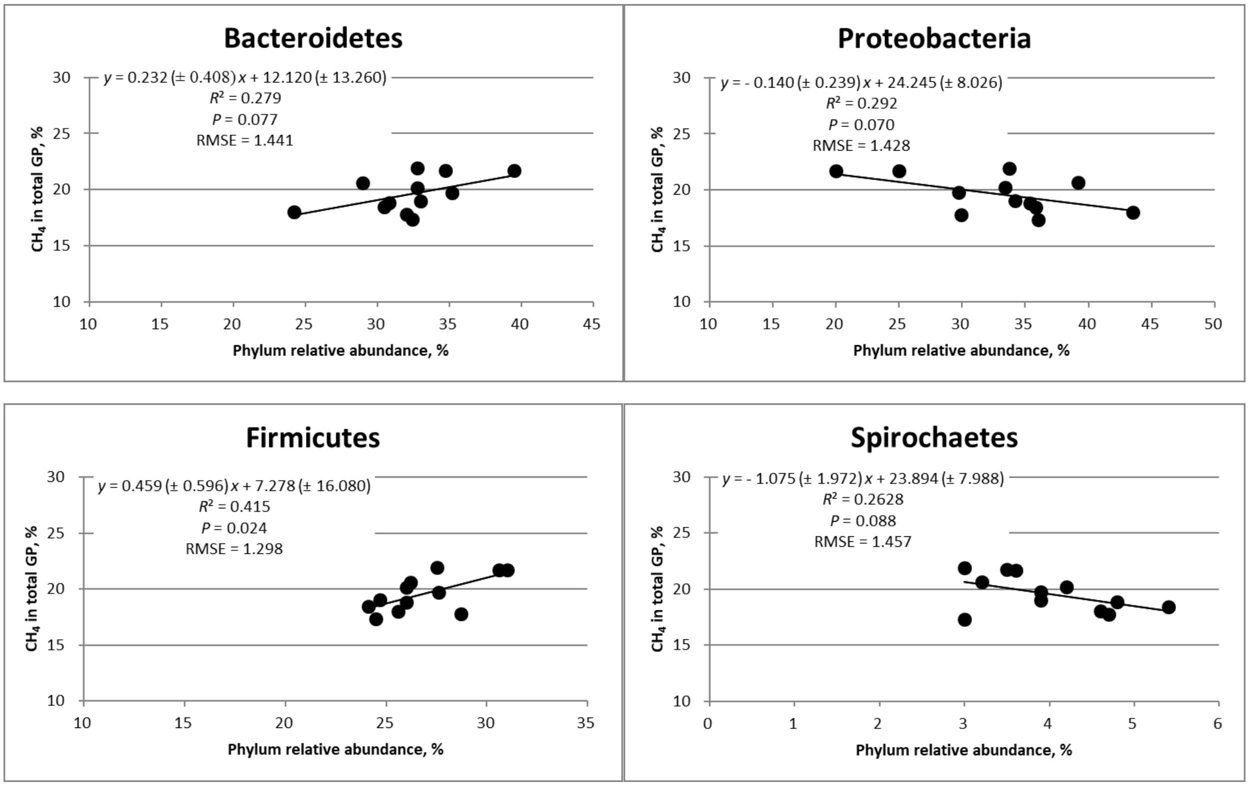
Treponema	2.96	1.75	1.77	3.09	0.829	0.437
YRC22	0.613	0.510	0.808	0.530	0.224	0.708

[1] CTR, control.

[2] BOR, bornyl acetate; CAM, camphor; EUCA, eucalyptol.

[a, b] Means in the same row with different superscripts are statistically different at $P < 0.05$

APPENDIX



Appendix Figure. Linear regression between main bacteria phyla relative abundance and CH_4 emission (% total GP). GP = gas production; RMSE = root mean square error.

Appendix Table. Correlations (Pearson coefficient) among protozoa and main Bacteria phyla and Euryarchaeota in the Exp. 1

Item	Euryarchaeota	Bacteroidetes	Proteobacteria	Firmicutes	Spirochaetes
Total protozoa	-0.526	-0.627*	0.646*	-0.709**	0.538
Entodinium	-0.584*	-0.630*	0.658*	-0.732**	0.530
Diplodinium	0.304	-0.105	-0.039	0.157	0.210
Ophryscolecinae	0.697*	0.442	-0.398	0.432	-0.377

*Significance at $P < 0.05$; ** Significance at $P < 0.01$.

4. Experiment 2

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The effect of the forage basis of the diet on methane emission and performance of dairy cows: a meta-analysis

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INTERPRETATIVE SUMMARY

Great effort has been posed in investigating the effects of dietary manipulation on enteric CH_4 emission from dairy cows. The forage basis can affect enteric CH_4 emissions and it is important to investigate the effect of forage basis on emission. The study provided a database of quantitative factors concerning diet composition and production performance in cows fed diets with different forage bases. These factors could be used to find prediction models of CH_4 emission, applicable also in a typical dairy farm considering the type of diet in terms of main forage composition.

ABSTRACT

The study aimed to compare the effect of the forage bases in the diet of dairy cows in terms of CH_4 emission and lactation performance through a meta-analysis approach. The study also investigated the correlation between enteric CH_4 and quantitative factors related to diet composition and performance in lactating cows. Models were proposed to predict enteric CH_4 (g/d, g/kg dry matter intake (DMI), and g/kg milk yield) with quantitative factors available in a typical dairy farm. A dataset was built collecting data from *in vivo* studies concerning CH_4 emission of dairy cows. Studies involving the use of feed additives or lipid supplementation were discarded. The treatment was assigned according to the forage most included in the diet (% DM) for each observation. Only diets based on corn silage (CS), alfalfa silage (AS), grass silage (GS), and fresh green forage (GF) were kept in the final dataset. Cows fed CS and AS had the highest DMI (21.9 and 22.0 kg/d) followed by GS (18.6) and GF (16.8); CS and AS also resulted in higher milk yield (29.7 and 30.4 kg/d) followed by GS (26.0) and GF (21.7). The NDF digestibility (%) was highest for GS and GF (67.6 and 73.1 respectively) than CS and AS (51.8 on average). Methane emission (g/d) was lower for GF (332) than the silage-based diets (on average 438). The CS and AS resulted in the lowest CH_4 /kg of milk yield (14.6 g/kg, on average), while for CH_4 kg/DMI the only difference was between CS and GS (19.7 vs. 21.3, respectively). Among the most important correlations tested, dietary NDF (%) was significantly correlated with CH_4 emission with a negative slope (-3.71), while the slope was positive in the correlations with CH_4/DMI (slope = 0.18) and CH_4/milk yield (slope = 0.27). However, the forage basis affected these correlations, and for CS the correlations were not significant. The correlation between CH_4/milk yield and NDFD was negative for GS (slope = -0.09), while it tended to be positive for CS (slope = 0.07). Milk fat concentration (%) was correlated with both CH_4/DMI (slope = 3.96) and CH_4/milk yield (slope = 3.51). Prediction model for emission was also obtained through a stepwise multi regression as follows: CH_4 (g/d) = - 65.3(±63.7) + 11.6(±1.67) × DMI - 4.47(±1.09) × diet CP (%) - 0.86(±0.33) × diet Starch (%) + 2.62(±0.78) × OM digestibility (%) + 30.8(±9.45) × Milk fat (%).

Key words (2-5): Silage, grazing, meta-analysis, predicting model, enteric methane

INTRODUCTION

Agriculture contributes to 38% of total anthropic CH_4 emissions, with enteric fermentation being the main cause representing 72% of the agriculture-produced CH_4 (Faostat, 2021). Diet chemical composition and digestibility can influence enteric CH_4 emission in the dairy cow; therefore, dietary manipulation was suggested as a practical approach for mitigating CH_4 emission in ruminants (Beauchemin et al., 2009). The diet of dairy cows is based on forages, and factors like forage to concentrate ratio and forage type have been investigated for their potential to affect rumen methanogenesis (Beauchemin et al., 2020). The NDF concentration of the diet is generally positively correlated with CH_4 emission because NDF fermentation gives a higher proportion of acetate, and thus a higher amount of hydrogen to be disposed of in the form of CH_4 (Moss et al., 2000). A recent study (Eugene et al., 2021) reviewed how the main characteristics of forages could impact CH_4 emission. The authors highlighted that the advancing stage of growth for grasses decreases forage digestibility, increasing CH4 yield and intensity. However, it is important to consider also other factors such as the family of the forage crop. For example, legume forages lead to lower CH_4 emission due to lower NDF concentration, higher DMI, and higher passage rate in the rumen than grass forages (Beauchemin et al., 2009). In the meta-analysis of Archimède et al. (2011), the authors evaluated the methanogenic potential of C4 (like kikuyu grass) vs. C3 (like ryegrass) grasses and cold vs. warm climate legumes in ruminant diets. In this study, legume forages were confirmed to be less methanogenic than grass. The results also showed that C4 grass forages were associated with 10 – 17% (depending on the unit) more CH_4 emission than C3. The authors explained the difference between C3 and C4 partly because of a change in rumen fermentation profiles (increase in acetate with advancing maturity for C4) and partly because of the longer retention time for C4, allowing higher digestibility and thus CH_4 emission.

Great effort was posed in understanding and quantifying the effect of dietary forage, like corn silage and alfalfa silage in the North American diets (e.g. Arndt et al., 2015; Liu et al., 2012), grass silage in the Nordic countries (e.g. Cabezas-Garcia et al., 2017; Hart et al., 2015), and pasture (e.g. Carmona-Flores et al., 2020) on CH_4 emission. However, to the best of our knowledge, only a few *in vivo* studies followed a comparative approach testing more than two different forage bases and the implications on CH_4 emission within the same experiment (i.e., Gunal et al., 2018; Gislon et al., 2020).-Therefore, a meta-analysis could be a practical approach to compare diets with different forage bases summarizing the results from different studies and considering the variability among studies. Recently, several meta-analyses investigated the effects of supplements on enteric *in vivo* CH_4 emissions, such as the dietary supplementation of nitrate (Feng et al., 2020), 3-nitrooxypropanol (Jayanegara et al., 2018), monensin (Appuhamy et al., 2013), lipid (Eugene et al., 2008), and essential oils (Khiaosa-ard and Zebeli, 2013). However, rare studies quantitatively evaluate the effects of forage types on CH_4 emission by meta-analysis. As far as we know, only the studies of Archimède et al. (2011) and Sauvant et al. (2014) built a dataset of *in vivo* experiments carried out without using rumen modulators. However, both these studies did not evaluate the effect of the single forage type on enteric CH_4 emission.

Given these considerations, the objectives of the present meta-analysis were to 1) compare CH_4 emissions and lactation performance of cows fed diets with a different forage basis; 2) evaluate the potential of variables related to diet characteristics and lactation performance in predicting CH_4 emissions.

MATERIALS AND METHODS

The literature review was conducted using PubMed (US National Library of Medicine, Bethesda, MD) and Web of Sciences (Thomson Reuters Science, New York, NY). The keywords used were “methane, dairy cows” with no publication date or language filter applied. A total of 2818 articles matched the query as shown in the PRISMA diagram (Figure 1), and were filtered according to the following criteria: only full paper *in vivo* studies were considered; CH_4 had to be measured by either respiration chambers, Greenfeed or SF_6; only studies with lactating dairy cows were included; no additives to modulate enteric CH_4 emission (i.e. added fats, nitrates, ionophores, tannins, saponins, or other CH_4 inhibitors) were included in the diet. After duplicates removal, these articles were scrutinized progressively by title, abstract, and full paper. Sixty-four papers passed this screening and were used to build the dataset for meta-analysis.

The main forages used as an experimental treatment in the present meta-analysis were: corn silage, alfalfa silage, winter cereal silage, grass silage, alfalfa hay, grass hay, and green forage (either grazed or fed right after cutting). For each study, the data of each experimental treatment were assigned to one of the previous categories according to the main forage inclusion level. Observations were discarded when treatment was not investigated in at least five studies or a diet contained two forages in the same concentration.

As a result, four forage treatments remained in the dataset: corn silage (**CS**), alfalfa silage (**AS**), grass silage (**GS**), and green forage (**GF**); the papers used are listed in Supplementary Table 1. Preliminary data screening was conducted on the variables CH_4 per kg of DMI (**CH_4/DMI**) and CH_4 per kg of milk (**CH_4/MY**): data which exceed 1.5 interquartile ranges (**IQR**) below the first quartile (first quartile - 1.5 IQR) or above the third quartile (third quartile + 1.5 IQR) were considered as an outlier, and thus the value was discarded (Upton and Cook, 1996). The number of observations for each forage treatment is reported in Table 1. The final dataset was built extracting data from each paper regarding the experiment (cows per treatment, duration), forage (inclusion in the diet), diet (forage to concentrate ratio, chemical analysis, and total tract digestibility), cows (breed, DIM, parity, BW), milk (production, quality, dairy efficiency), DMI, and CH_4. The data about CH_4 regarded **CH_4 emission** (g/d), **CH_4 yield** (g/kg DMI), **CH_4 intensity** (g/kg milk yield), and the conversion factor of gross energy intake (**GEI**) into CH_4 (**Y_m**). When CH_4 was reported in liters, it was converted in grams assuming a molar gas volume of 22.4 L and a molar weight of 16.0 g. When not reported in the paper, Y_m was calculated by dividing the energy lost as CH_4, considering a conversion of 55 kJ/g of CH_4, per the GEI of the cow. When GEI was not available, it was calculated using the equation of Ewan (1989):

$$\mathrm{GEI}\ (\mathrm{kJ/kg\ DM}) = (17350 + (234.46 \times \mathrm{EE}) + (62.8 \times \mathrm{CP}) - (184.22 \times \mathrm{Ash})) \times \mathrm{DMI}$$

with crude protein (CP), ether extract (EE), and Ash expressed as % of DM.

Statistical analysis was performed using Proc Mixed of SAS, version 9.4 (SAS Institute, Cary, NC). The observations for dependent variables were weighted by the inverse of the squares of each mean SE (St-Pierre, 2007), using the weight statement of SAS. The study effect was assumed as a random effect (St-Pierre, 2007). The unstructured variance was applied to the model, using the "type = un" option (unstructured covariance) in the random statement with "subject = study".

Data were analyzed on three levels. The first one was to evaluate the effect of different forages on the DMI, animal performance, CH_4, and diet chemical composition with the following model:

$Y_{ij} = \mu + S_i + F_j + \varepsilon_{ij}$

where Y_{ij} is the dependent variable; μ is the overall mean; S_i is the random effect of the study; F_j is the forage treatment effect; ε_{ij} is the residual error of the model.

In the second level, different correlations were tested to evaluate the relationship between CH_4 emission with other variables (diet chemical composition, milk production, and DMI). First, the whole dataset was considered, and then it was divided based on the forage types. The following model was applied:

$Y_{ij} = B0 + S_i + B1\ X_{ij} + b_i\ X_{ij} + e_{ij}$

where Y_{ij} is the dependent variable; B0 is the overall (inter-study) intercept; S_i is the random effect of the study; B1 is the overall regression coefficient of Y on X; X_{ij} is the value of the continuous predictor variable; b_i is the random effect of study on regression coefficient of Y on X; e_{ij} is the residual error.

The stepwise method was also applied to predict CH_4 emission. All possible explanatory variables (inclusion of the main forage, forage to concentrate ratio, diet composition and total tract digestibility, milk composition and production, DMI) were included in the initial model. The dependent variables were CH_4 emission (g/d), yield (g/kg DMI), and intensity (g/kg milk yield). Only the variables that showed significant effects ($P < 0.05$) were kept in the final model. The model was:

$Y_{ij} = B0 + B1\ X1_{ij} + b0 + b1\ X1_{ij} + B2\ X2_{ij} + \ldots + Bn\ Xn_{ij} + e_{ij}$

where Y_{ij} is the dependent variable; B0 is the overall (inter-study) intercept; B1, B2, … Bn are fixed regression coefficients of Y on independent variables $X1_{ij}$, $X2_{ij}$,…Xn_{ij}; b0 and b1 are the random effects of study i on the intercept and regression coefficient of Y on X1 in study i; eij is the residual error. Multicollinearity was evaluated through variance inflation factor (VIF); the variables having VIF > 10 were removed from the final model in order to exclude highly correlated variables in the same model (Vittinghoff et al., 2012). Finally, the models were evaluated based on Akaike's information criterion with correction (AICc).

For all of the three levels, significance was declared at $P \leq 0.05$ and tendency as $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Effect of different forage on dry matter intake and milk production

The chemical composition of the diets was different depending on the forage treatment (Table 2). As expected, the DM of GF was lower than the silage-based diets (31.9 vs. 50.2%, for GF and for the average of CS, AS, and GS, respectively, $P < 0.05$). The concentration of NDF (% DM) was the highest for GF (43.1), followed by GS (38.2), while no difference was found between CS and AS (34.7 and 31.8, respectively) ($P < 0.01$). Dietary NDF reflected the NDF concentration of the main forage included, with alfalfa and corn silages containing less fiber than grasses (Dewhurst, 2013). Starch concentration (on DM) was higher for CS and AS (23.6 and 20.6%, respectively) than GS and GF (12.2 and 9.86%, respectively) ($P < 0.01$). The other two nutrients affected by the treatment were OM ($P < 0.01$), with CS (93.8% on DM) having the highest concentration, and CP ($P < 0.01$), with GF having the highest concentration (18.7% on DM). The highest CP concentration of GF was expected since pasture proved to be a CP-rich forage (Waghorn et al., 2016; Wims et al., 2010).

Cows fed CS and AS had lower NDF digestibility (**NDFD)** (49.9 and 53.6%, respectively) compared to GS and GF (67.6 and 73.1%) ($P < 0.01$) and the NDFD was negatively correlated with dietary starch concentration; de Souza et al. (2018) found a linear effect of starch on NDFD: every 1% increase in dietary starch corresponded to a reduction of 0.59% NDFD. In the present study, the correlation between starch and NDFD correlation was significant (P < 0.01), with a slope = -0.63 and comparable to that found by de Souza et al. (2018). Only a few studies in the present dataset reported the values of NDFD of the dietary forages, but it can be assumed that corn and alfalfa silages had similar NDFD and lower than grass silage. This hypothesis is supported by Pirondini et al. (2015), where corn and alfalfa silages had similar values of NDFD (48.3 vs. 48.2%, 48 h in situ incubation), significantly lower than Italian ryegrass silage (75.1%). Regarding GF, several factors may affect NDFD (species composition, sampling date, season, etc.), which is generally high, as reported by Jensen et al. (2016). On average, the authors found values of NDFD, ranging from 78.8% of perennial ryegrass to 65.2% of Sandberg bluegrass.

Cows fed the GF diet had lower DMI and milk yield (16.8 kg/d and 21.7 kg/d, respectively) compared to the silage-based diets (*P* < 0.05) (Table 2). This result was expected because of higher inclusion of forage in the diet and higher NDF concentration (on average 83% of forage proportion on DM basis for GF compared to 52, 48, and 58%, for CS, AS, and GS, respectively) which are both negatively correlated with DMI (Ben Meir et al., 2021). Dietary starch concentration also might affect DMI, with a higher level of DMI for higher starch diets (i.e. CS and AS), as also found by Ipharraguerre et al. (2002) for dairy cows. Dewhurst (2013) also suggested that the higher intake of forage like corn and alfalfa silages, compared to grass silage, could be due to faster fermentation and physical breakdown in the rumen. In the review of Khan et al. (2015), the inclusion of corn silage into grass silage-based diets improved DMI of 1.55 kg/d and milk yield of 1.91 kg/d, in agreement with the present meta-analysis. The similar results of DMI, milk yield, and dairy efficiency between CS and AS in the present meta-analysis are in agreement with Arndt et al. (2015) but in contrast with the study of Uddin et al. (2020), which found higher DMI and milk production for cows fed diets based on corn silage in comparison to diets based on alfalfa silage. In Uddin et al. (2020), the different production performances were probably due to lower fiber digestibility for diets based on alfalfa silage than corn silage which was not observed in the present dataset.

The differences in DMI between diets based on different forage types (CS and AS > GS > GF) were reflected in milk production, with CS and AS having the higher milk yield (29.7 and 30.4 kg/d, for CS and AS, respectively,); GS was intermediate (26.0 kg/d), and GF was the lowest (21.7 kg/d) (*P* < 0.05), confirming the positive correlation between DMI and milk yield (Pearson correlation coefficient between DMI and milk yield = 0.86). This value was higher than that (0.52) obtained by Hristov et al. (2005) in a meta-analysis. Despite the difference in DMI and milk yield, dairy efficiency was not affected by the forage treatment (*P* = 0.29). Dairy efficiency is negatively correlated with NDF concentration and forage to concentrate ratio (Britt et al., 2003) that were affected by the forage treatment in the present meta-analysis. However, higher NDFD for GF and GS (the diets with the highest NDF concentration) might have compensated for these effects since improved digestibility causes great improvements in milk yield rather than in DMI (Oba and Allen 1999).

Effect of different forage on methane emission

The CH_4 emission (g/d) was affected by the forage type, and the silage-based diets had a higher CH_4 emission compared to GF (439, 450, 425, and 332 g/d for CS, AS, GS, and GF, respectively; *P* < 0.05) as a consequence of the different DMI, which is strongly correlated to CH_4 emission (Table 2). The present results partially agree with the findings of van Gastelen et al. (2019). In this latter review, a variety of mitigation strategies for different ruminant species was studied. Among these strategies, the partial or complete substitution of pasture, grass silage, or alfalfa silage with corn silage caused an increase of DMI and CH_4 emission (+ 8%) for dairy cows. In contrast to van Gastelen et al. (2019), the inclusion of CS did not increase CH_4 emission compared to GS and AS treatments but only compared to GF. On the other hand, in the study mentioned above (van Gastelen et al., 2019), increased legume forages at the expense of pasture also caused increased DMI and CH_4 emission, in agreement with the present study.

However, when CH_4 yield (g/kg DMI) is considered, GS gave the highest value (22.3 g/kg), even if not statistically different from AS and GF (20.8 and 21.3 g/kg, respectively), while CS (19.7 g/kg) was numerically the most efficient treatment although not different than AS and GF. The difference between the CH_4 yield of CS and GS (P < 0.05) was probably due to higher DMI for cows fed CS diets and the lower NDF concentration of the diet. In the review of van Gastelen et al. (2019), the inclusion of corn silage in the diet of dairy cows decreased CH_4 yield (- 5%) compared to alfalfa silage, grass silage, and pasture. In the present study, CS had a -11.7% CH_4 yield than GS, whereas the difference with AS and GF was -5.3 and -7.5%, respectively, although not significant. Regarding the comparison between CS and GF, the results are in agreement with Civiero et al. (2021) (no difference in CH_4 yield according to the different proportion of TMR, based on corn silage and green forage) and in contrast with Dall-Orsoletta et al. (2016) and O'Neil et al. (2011) who showed that cows fed corn silage based TMR had higher CH_4 yield than cows fed a TMR supplemented with pasture or total pasture. The cows fed GF had higher NDFD and OMD than CS, and it has been shown that increased digestibility of feeds results in less CH_4 being emitted per kg of DMI (Blaxter and Clapperton, 1965), as shown in the present study where CS and GF diets were not different for CH_4 yield.

There was a significant difference among diets for CH_4 intensity (g/kg of milk yield): GF had the highest intensity (18.2 g/kg), and CS had lower intensity (14.2) than GS (15.9 g/kg), AS (14.9) was similar to CS and GS. The starch content may explain the differences among diets for intensity since several authors found differences in CH_4 intensity according to the starch concentration of the diets (Aguerre et al., 2011; Hassanat et al., 2013; Hatew et al., 2015). Starch fermentation in the rumen favors propionate production rather than acetate (Bannink et al., 2006), representing an alternative hydrogen sink competing with CH_4. Moreover, part of the starch (6-78%; Moharrery et al., 2014) may reach the intestine without being digested in the rumen, and this reduces the amount of OM fermented in the rumen, which is highly correlated with CH_4 emission (Sauvant et al., 2011). Moroever, Van Kessel and Russell (1996) showed that rumen fluid pH is reduced with high grain (i.e. high starch) diets, creating a ruminal environment less favorable for methanogens, up to a point (i.e. pH < 6) wherein in vitro methanogenesis was totally inhibited. Similarly, Van Lingen et al. (2016) confirmed a threshold of pH value under that methanogenesis could not proceed further. In addition, milk yield increases with starch-rich diets, decreasing the intensity of CH_4 emission due to the lower relative amount of CH_4 referred to maintenance (Hassanat et al., 2013).

The Y_m (CH_4 energy loss as a percentage of GEI) was another variable considered in the present study, and it is a key variable used by IPCC (2019) to estimate enteric CH_4 emission from ruminants. In the present study (Table 2), this value was affected by the forage basis ($P < 0.01$), with CS and AS having a lower Y_m (6.01%, on average) compared to GS and GF (7.13% on average). Similarly, in Gislon et al. (2020), diets based on corn or alfalfa silages had similar Y_m values, while Hart et al. (2015) found that diets based on corn silage had lower Y_m compared to diets based on grass silage. On average, the value obtained (6.59%) is higher compared to the one suggested by IPCC (2019) for high-yielding cows (5.7%) considering the whole IPCC dataset.

Correlation of methane emission with diet characteristics

Table 3 and Table 4 present different correlations between CH_4 and quantitative factors related to diet chemical composition, total tract digestibility, DMI, and production performance considering the whole dataset (Table 3) or the dataset for each forage basis (Table 4). Only the most significant correlations and only the CS, GS, and GF results are presented due to the limited number of observations for AS (n = 8).

Methane emission (g/d) was correlated with diet OM and NDF concentrations (% DM) (Table 3). A positive correlation was found for CH_4 and OM ($P < 0.01$), while the correlation between CH_4 and NDF was negative ($P < 0.01$). The fermentation of fiber in the rumen is related to higher acetic acid production, which should increase CH_4 emission, in contrast with the present results. However, dietary NDF negatively influences DMI, the main driver of CH_4 production. The negative correlation between CH_4 emission and dietary NDF was confirmed when the single forage type was considered (Table 4) but with different results. The CH_4 emission was negatively correlated with dietary NDF (% DM) for GS and GF (slope = -1.25 and -3.86, respectively). For these two diets, DMI was negatively correlated to NDF (%) (slope = -0.12; $P < 0.01$ for GS; slope = -0.11; $P < 0.01$ for GF) explaining the negative relationship between CH_4 emission and fiber content. In contrast, for CS no significant correlations between CH_4 emission and dietary NDF and between DMI and NDF were observed. Ramin and Huhtanen (2013) observed that dietary carbohydrate composition showed only marginal effects on CH_4 production and our results confirm the effect of forage basis on CH_4 emission mainly for an effect on DMI and not for NDF content.

Similarly, the starch concentration of the diet (Table 3) only tended to decrease CH_4 emission ($P = 0.07$), but the effect was not significant, although the main forage of the diet affected the relationship. Hassanat et al. (2013) demonstrated that no effect on CH_4 production was found when starch content incrased from 17 to 22.8 % of DM in dairy cattle, but a further increase to 30% did reduce CH_4 production.

Interestingly, in the present meta-analysis, considering the range of starch for each diet, the CS and GF diets had the maximum starch content and higher than the threshold value reported by Hassanat et al. (2013) (32.6 and 32.8% on DM respectively for CS and GF), while the upper range value (25.0%) was lower for GS. For GS diet, no significant relationship was observed between CH_4 emission and dietary starch content differently from CS and GF diets with a negative and significant ($P < 0.05$) relationship.

Significant correlations were found between CH_4 yield (g/kg DMI) and OM, NDF, starch, CP (% DM) and NDFD (%) (Table 3). Methane yield was positively correlated with OM (slope = 0.55, $P < 0.01$) and NDF (slope = 0.18, $P < 0.01$) whilst starch was negatively correlated with CH_4 yield (slope = -0.12, P < 0.01). Considering the forage basis, the correlation with dietary starch was significant for CS and GF, (slope -0.19 and -0.22, respectively) but not for GS (Table 4), in agreement with the results about CH_4 emission.

A negative correlation was found between CH_4 yield and dietary CP (slope = -0.36, $P < 0.01$), and, according to the literature, this correlation is controversial (Hynes et al., 2016; Sauvant et al., 2011). Hynes et al. (2016) ran an experiment where diet concentrations of NDF and starch were nearly the same, but CP varied. They found that the effect of CP level of diet was not significant for any way to express CH_4 production, probably because of the low range of CP within the 3 diets (from 16.9 to 18.3%). In Niu et al. (2016), two different levels of CP (i.e. about 15% and 18.5%) in diets based on alfalfa hay did not affect CH_4; thus, the authors concluded that dietary CP concentration was a negligible factor in CH_4 emission. In the present meta-analysis, the range of dietary CP was high (from 8.17% to 27.5%, data not shown), and this might have contributed to a significant correlation between CH_4 yield and CP. Some authors also asserted that the effect is rather indirect due to concomitant change in the percentage of other nutrients into the diet (i.e. NDF and starch; Hynes et al., 2016). This hypothesis could explain the significant correlations found when considering the single forage type: the correlation between CH_4 yield (g/kg DMI) and CP was significant and negative for GS and GF ($P < 0.05$) (Table 4), the diets with the wider range for CP content (% DM) (11.8 and 25.1 for GS; 8.17 and 27.5 for GF *vs.*13.8 and 18.5 for CS). Overall it is important to notice a possible trade-off between CH_4 emission and N losses into the environment because most of the strategies to reduce N excretion are based on decreasing dietary CP. As a result, higher CH_4 emission can be observed when rumen fermentable carbohydrates substitute CP (Dijkstra et al., 2011). This aspect is important for diets based on grass silages or pasture rather than for diets based on corn silage which usually have a narrow range of CP content.

The CH_4 yield was also positively correlated with NDFD ($P = 0.02$, Table 3). Increased NDFD favors CH_4 production because it leads to higher acetate production and increases the pool of OM digested in the rumen, in agreement with the study of Ramin and Huhtanen (2013), which showed a positive correlation between CH_4 yield and OMD. However, also for this relationship, an effect of the main forage basis was observed with CH_4 yield not significantly correlated to NDFD for GS diet. The NDFD is an indicator of forage quality, and it is usually associated with grass forage CP content: the higher the CP content and the highest the NDFD digestibility, as observed by Cabezas-Garcias et al. (2016). As found by Warner et al. (2016) and confirmed by data of the present dataset, experimental treatments leading to higher CP content in grass silage decreased CH_4 yield. This effect may have counterbalanced the expected relationship between CH_4 yield and NDFD for GS.

Regarding CH_4 intensity (g/kg milk yield), the significant correlations ($P < 0.01$) were with dietary NDF and starch (slope = 0.27 and - 0.11, respectively), highlighting the important effect of these two nutrients on both CH_4 and milk production (Table 3). However, the forage basis affected the correlation between CH_4 intensity and dietary NDF with a positive correlation ($P < 0.01$) for GS and GF (Table 4) while for CS it was found only a tendency ($P = 0.06$), but the correlation was negative (slope = -0.17). For GS and GF milk production was negatively correlated with dietary NDF *(*$P < 0.01$, data not shown), thus explaining the negative relationship between intensity and dietary NDF. However, it is worth underlining that for these two diets increasing NDFD increased significantly ($P < 0.01$) milk production (on average 0.17 kg more milk produced for an increase of one point of NDFD). For this reason, for GS the correlation between CH_4 intensity and NDFD was negative and significant (slope = -0.09, $P = 0.03$) confirming the importance of feeding cows with high-quality fiber forages. For GF, the number of observations was not enough (n=7) to have a robust dataset. On the other hand, for CS no relationship was observed between intensity and NDFD.

The negative tendency between CH_4 intensity and NDF found for CS could be explained considering the lower NDFD of corn silage compared to grasses (Khan et al., 2015; Pirondini et al., 2015) and the higher passage rate and intake of corn silage (Krizsan et al. 2010) than grass which might limit the amount of fiber fermented in the rumen and thus CH_4 emission. Although it was not investigated in the present meta-analysis, it can also be speculated that cows fed corn silage diets also had a different rumen microbiome than cows fed grass diets. For example, Deush at al. (2017) showed that a higher abundance of Proteobacteria and Succinivibrionaceae were observed in the rumen of cows fed corn silage and the increased use of hydrogen by succinate producing bacteria can partially explain lower CH4 emissions from dairy cows fed corn silage as compared to GS diets (Vaidya et al., 2020).

The CH_4 intensity also depended on the starch content of the diet but, considering the forage basis, the correlation was significant ($P = 0.01$) only for GF with a negative slope. The slope between milk production (kg/d) and dietary starch (% DM) was significant ($P < 0.01$; slope = 0.21) only for GF whit decreased methane intensity as a result of greater milk yields at the higher starch levels. On the other hand, for CS an increase in starch level was not associated with an increase in milk production in agreement with other studies (Gislon et al., 2020; Powell et al., 2016), which demonstrated that feeding more corn is not related to any productive advantage for the animals. The same was observed for GS in the present dataset (no correlation with dietary starch) suggesting that increasing NDFD is more important for milk production than increasing starch content for grass silage-based diets.

Correlation of methane emission with DMI and lactation performance

Methane emission (g/d) was positively correlated with DMI and milk yield ($P < 0.01$) (Table 3). The slope of the correlation between CH_4 and DMI (kg/d) (14.1) was in line with the ones found by Hristov et al. (2018) and Niu et al. (2018): 13.6 and 13.3, respectively. Instead, the slope for the regression between CH_4 and milk yield (kg/d) was higher in the present study: 5.41 vs. 2.54 and 2.73 for Hristov et al. (2018) and Niu et al. (2018), respectively.

Considering the relationship for each forage category, CH_4 emission and DMI were positively correlated, but the correlation was not significant for CS ($P = 0.33$) differently than the other treatments (Table 4). The reason may be the high starch concentration of corn silage (highly degradable), which can act as a confounding factor: higher DMI of a diet based on corn silage means higher intake of starch (more propionic acid and less methane for the unit of fermented starch), which might outbalance the increase in CH_4 emission due to DMI. The study of Hassanat et al. (2013) can be considered to support this hypothesis. In this study, when corn silage was the only forage basis in the diet, CH_4 emission was not different compared to the treatment based solely on alfalfa silage, despite an increase in DMI for the corn silage diet.

The correlation between CH_4 emission and milk yield was positive for all the diets (Table 4) but, unexpectedly, significant only for GS and with a higher slope value than CS and GF ($P < 0.01$). The different starch intake might explain this difference due to the negative correlation between CH_4 emission and starch concentration (% DM) for CS and GF differently from GS. Starch increased milk yield but, probably, not as much CH_4 emission; whereas, unexpectedly, milk production for GS was not sensitive to changes in dietary starch.

Significant ($P < 0.01$) correlations were found between CH_4 yield and milk yield and milk fat concentration (Table 3). The correlation between CH_4 yield and milk production was negative. This result confirmed that increasing the milk production level could dilute the environmental effects of enteric CH_4 in dairy cows (Harper et al., 2018; Monteny et al., 2006; Uddin et al., 2020).

Milk fat (%) and CH_4 yield (g/kg DMI) were positively correlated because they are both linked to rumen acetate (Moate et al., 2017; 2018). A diet that leads to lower acetate production reduces the acetate concentration in the rumen, with a negative effect on both CH_4 and de novo milk fat synthesis (Requena et al., 2020).

Methane intensity (g/kg milk yield) was significantly correlated with DMI, milk fat, and protein concentrations. The correlation with DMI was negative ($P < 0.01$), suggesting that an increase in DMI is more effective on milk yield than on CH_4 emission (g/d), as observed in Arndt (2021) as well: an increase of +58% for DMI caused an increase of +18% for CH_4 emission, of +17% for milk yield, but a reduction of CH_4 intensity of -17%. The correlation with milk fat was positive ($P < 0.01$) considering the whole dataset and each forage basis. Besides the biological link between milk fat and CH_4 discussed above, this result, together with the positive correlation between CH_4 intensity and milk protein ($P = 0.03$), could be due to the negative correlation between milk composition and milk yield. In the meta-analysis of Huhtanen et al. (2007), the authors found that increasing milk yield was associated with lower milk fat concentration. Thus, higher milk fat concentration is associated with lower milk yield and, thus, higher values of CH_4 intensity. Similarly, Morton et al. (2016) found a negative association between milk protein and milk yield; thus, the higher the protein, the lower the milk yield, and thus the higher the ratio CH_4 on milk yield. These results suggested a dilution effect of milk components with higher milk yield, and thus, a dilution of CH_4 intensity as hypothesized from the present study results.

Multiple regression between methane and diet composition and lactation performance

The models derived from the multiple stepwise regression are in Table 5: quantitative factors related to diet characteristics, DMI and lactation performance, were used to predict CH_4 emission (g/d), yield (g/kg DMI), and intensity (g/kg milk yield).

The CH_4 emission (g/d) was positively correlated with DMI, OMD and milk fat, and negatively correlated with dietary starch and CP concentrations. Similarly, in the multiple regression of Velarde-Guillén (2019), the CH_4 best fit model also included DMI and milk fat concentration, which positively correlated with CH_4 emission. In the work of Santiago-Juarez et al. (2016), only quantitative variables potentially available in a dairy farm were considered for prediction models of CH_4 emission. The best results from the above-cited study were obtained when DMI, milk yield and fat composition, diet composition, and BW were included in the model.

Similar to emission results, the CH_4 yield was positively correlated with OMD and milk fat concentration and negatively with dietary CP. In the multiple regression proposed by Ramin and Huhtanen (2013) for CH_4 emission, DMI and OMD were included as well, with positive slopes; furthermore, OMD was positively correlated with CH_4 yield as observed in the present study.

The CH_4 intensity was negatively correlated with DMI and the inclusion (% of total diet DM) of the main forage in the diet and positively with forage to concentrate ratio, dietary OM and NDF concentrations, and milk fat and protein concentrations. Similarly, Arndt et al. (2021) found that forage to concentrate ratio was positively associated with CH_4 intensity. Aguerre et al. (2011) found that increasing proportion of forage in the diet statistically increased CH_4 emission, yield and intensity. In agreement with these observations, Ferris et al. (2020) asserted that a higher level of concentrate in the diet could significantly affect milk production, diluting CH_4 per kg of milk. However, unexpectedly and contrary to forage concentrate ratio, the percentage of inclusion of the main forage in the diet was negatively correlated with CH_4 intensity, confirming a role of the single main forage of the diet in affecting CH_4 intensity. The reason might be that the main forage investigated in the studies of the present dataset proved to be of great nutritional quality (corn silage for the high energy content due to high starch concentration, grass silage and green forage for the high NDFD) sustaining high milk production. Probably the other fibrous feedstuff included in the diet was not excellent like the main forage, and thus they favoured CH_4 emission rather than milk production.

The results of the present study confirmed that one of the most important variables related to methane emission is DMI. The role of DMI was also underlined by Benaouda et al. (2019): among the models investigated in this paper for the prediction of CH_4 emission, the ones with the smallest root mean square prediction error were the ones including DMI as a predictor. In the present study, DMI was correlated both with CH_4 emission (positive slope) and intensity (negative slope). Dry matter intake is related to the amount of feed that can be fermented (Knapp et al., 2014), and the OM digested had crucial importance in determining CH_4 emission (Sauvant et al., 2011), as discussed above. The DMI contributed negatively to CH_4 intensity because it affected milk yield more than CH_4 emission, as previously reported (see Table 3).

Milk fat concentration, a parameter easily measurable in a dairy farm, was positively correlated with CH_4 in all three models obtained. The relationship between milk fat and CH_4 emission was investigated in several other studies. For example, the inclusion of milk fat concentration improved the performance of the prediction model for CH_4 emission in Moraes et al. (2014). Moreover, when milk fat concentration was included in a model considering only DMI as a predictor of CH_4 emission, the mean bias was halved in the study of Santiago-Juarez et al. (2016). These results were confirmed in the work of Williams et al. (2014) that found positive correlations between milk fat and CH_4 yield and intensity and between milk protein and CH_4 intensity. Given these findings, the authors suggested carefully evaluating genetic selection programs aimed at obtaining lower-emitting cows, because CH_4 appeared to be positively correlated with milk quality, thus having negative economic consequences for farmers.

The biological connection between the other factors of these models, namely starch (model 1), OMD (models 1 and 2), OM and NDF (model 3), and CH_4, DMI and milk yield has been widely discussed above. In particular, the contribution of starch (slope = -0.86) in the first model was lower than the other predictors, in agreement with Ramin and Huhtanen (2013), the authors found that dietary NFC (expressed as % of total carbohydrates) had an only marginal effect on CH_4 emission.

CONCLUSIONS

The results of the present meta-analysis confirmed that the dietary forage basis has an important impact on performance and CH_4 emission. All the forages implied in the studies were characterized by excellent quality: corn silage and alfalfa silage sustained high milk production, lowering the impact of CH_4 emission per kg of DMI or milk yield. Instead, multi-annual/perennial forages, like pasture and grassland, either grazed or harvested, had high values of NDFD. These forage bases have other environmental advantages compared to corn silage, like increasing soil carbon, another GHG mitigation strategy.

The importance of considering the different forage basis of the diet was also confirmed by the correlations tested: the quantitative factors investigated as a proxy of CH_4 emission performed differently according to the forage treatment. Therefore, CH_4 factors are suggested for each forage basis rather than fixed values. Finally, the multiple-regression models could also be useful tools for estimating CH_4 emission in a typical dairy farm since most of the predictors required are routinely available to farmers (i.e. milk production and milk quality analysis; diet chemical composition). However, these models should be validated on an independent dataset. This would require more studies on the subject. More studies are also needed to investigate other forage bases, widely used in dairy farms but not considered in this meta-analysis (e.g. winter cereal silages and hays) due to the paucity of observations.

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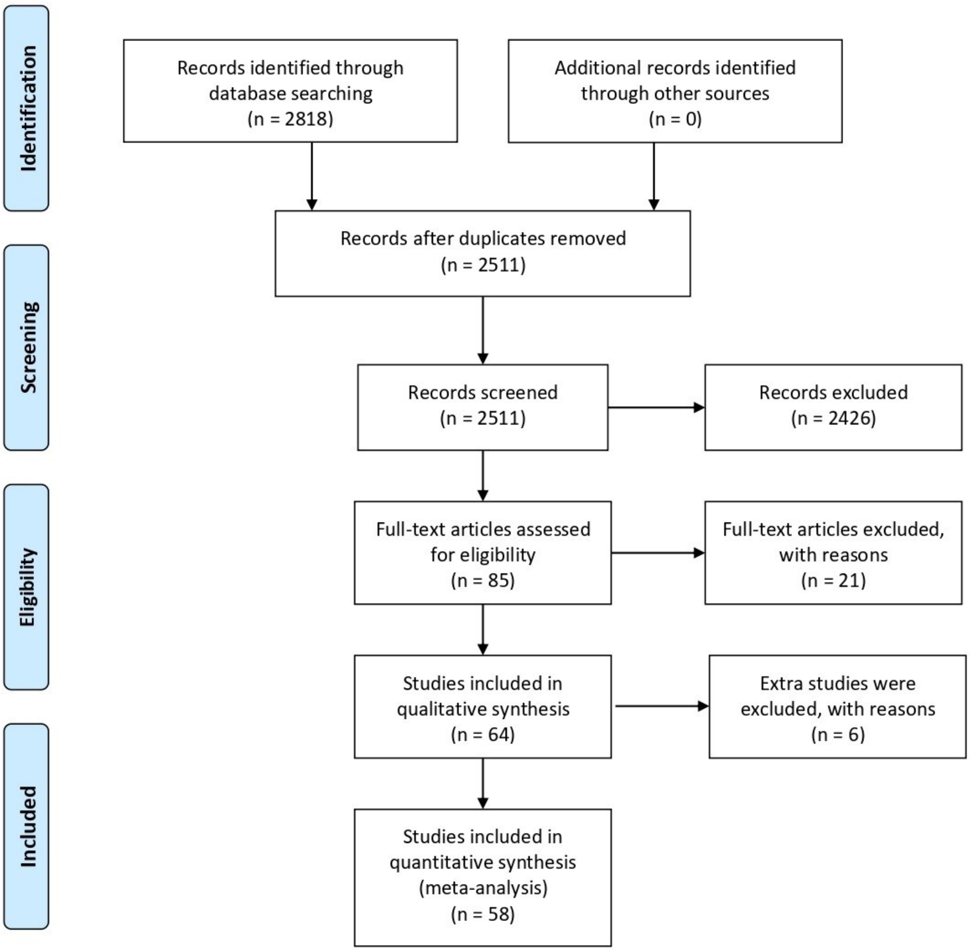

Figure 1: Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. (Moher et al., 2009)

Table 1. Descriptive statistics of the dataset (CS corn silage; AS alfalfa silage; GS grass silage; GF green forage)

	CS			AS			GS			GF		
Item	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD
Enteric CH_4												
CH_4 (g/d)	57	431	83.1	8	504	128	72	417	47.5	63	341	67.4
CH_4 yield (g/kg DMI)	56	19.5	3.28	8	21.7	3.12	70	22.9	2.08	62	21.6	3.72
CH_4 intensity (g/kg milk yield)	48	13.6	2.48	7	16.1	2.24	65	16.0	2.03	56	18.1	6.45
Diet composition, %												
DM	28	49.9	9.39	3	55.3	0.95	45	48.9	11.2	27	32.6	14.3
OM	39	93.5	1.12	8	92.1	1.38	55	92.2	1.39	52	91.5	1.89
NDF	53	35.0	4.19	7	29.0	1.35	61	39.0	4.97	56	44.3	8.74
Starch	39	23.8	4.57	7	21.2	3.70	53	12.3	7.69	13	10.4	8.36
CP	54	16.0	1.12	8	17.0	0.79	61	17.4	2.58	56	18.1	4.14
Inclusion in the diet, % DM	57	52.4	13.0	8	48.3	11.1	72	58.2	13.2	62	83.0	16.8
Total tract digestibility, %												
DM	20	69.9	3.40	7	71.4	1.37	19	72.3	3.71	11	78.1	2.68
OM	24	71.8	3.22	7	73.3	1.47	39	74.9	3.92	27	77.5	5.67
NDF	24	50.0	7.52	7	51.6	3.97	36	68.7	6.81	7	74.0	6.68
CP	21	68.8	3.61	7	69.2	5.17	23	67.5	4.89	0	.	.
Animal performance												
Milk yield, kg/d	52	31.6	7.12	7	32.3	5.42	72	25.5	3.93	57	21.4	5.34
DMI, kg/d	56	22.0	3.27	7	22.8	2.06	72	18.4	2.32	64	16.0	2.58
BW, kg	54	619	59.2	8	625	78.3	58	622	46.2	61	515	61.8
Milk composition, %												
Protein	51	3.31	0.26	7	3.15	0.21	64	3.47	0.27	55	3.45	0.21
Fat	51	4.19	0.60	7	4.14	0.49	64	4.49	0.39	55	4.41	0.64

Table 2. Dry matter intake, lactation performance and methane production of cows fed diets with different forage basis (CS: corn silage; AS: alfalfa silage; GS: grass silage; GF: green forage)

Forage	Unit	CS	AS	GS	GF	SE	*P* - value
CH_4	g/d	439^{a}	450^{a}	425^{a}	332^{b}	18.6	<0.01
CH_4 yield	g/kg DMI	19.7^{b}	20.8^{ab}	22.3^{a}	21.3^{ab}	0.98	<0.01
CH_4 intensity	g/kg milk yield	14.2^{c}	14.9^{bc}	15.9^{b}	18.2^{a}	1.30	<0.01
Y_m[1]	% of GEI[2]	5.89^{b}	6.22^{b}	6.97^{a}	7.28^{a}	0.39	<0.01
DM	% of AF	48.8^{a}	51.9^{a}	49.9^{a}	31.9^{b}	5.45	0.02
OM	% of DM	93.8^{a}	91.9^{b}	92.2^{b}	91.5^{b}	0.54	<0.01
NDF	% of DM	34.7^{c}	31.8^{c}	38.2^{b}	43.1^{a}	1.93	<0.01
Starch	% of DM	23.6^{a}	20.6^{a}	12.2^{b}	9.86^{b}	2.75	<0.01
CP	% of DM	15.9^{b}	16.8^{b}	17.1^{b}	18.7^{a}	0.89	<0.01
EE	% of DM	3.36	4.30	3.27	3.73	0.49	0.20
OMD[3]	% of OM	71.5	72.5	74.2	77.7	1.62	0.06
NDFD[4]	% of NDF	49.9^{b}	53.6^{b}	67.6^{a}	73.1^{a}	4.38	<0.01
DE[5]	% of GEI[2]	69.9	70.0	72.3	74.4	1.64	0.11
Milk yield	kg/d	29.7^{a}	30.4^{a}	26.0^{b}	21.7^{c}	2.39	<0.01
DMI	kg/d	21.9^{a}	22.0^{a}	18.6^{b}	16.8^{c}	1.13	<0.01
Dairy efficiency[1]		1.37	1.36	1.41	1.31	0.05	0.29
Milk protein	%	3.38	3.29	3.37	3.40	0.07	0.49
Milk fat	%	4.21	4.30	4.41	4.22	0.11	0.19
Milk urea	mg/dL	25.4	26.5	27.8	24.0	2.71	0.61

[1] Y_m: Methane conversion factor $= \frac{\text{energy lost as methane}}{\text{gross energy intake}} \times 100$

[2] GEI: Gross Energy Intake (kJ) $= \left(17350 + (234.46 \times \text{EE}) + (62.8 \times \text{CP}) - (184.22 \times \text{Ash})\right) \times$ kg DMI; with CP, EE, and Ash expressed as % of DM (Ewan, 1989)

[3] OMD: Total tract OM digestibility $= \frac{\text{digestible OM}}{\text{OM intake}} \times 100$

[4] NDFD: Total tract NDF digestibility $= \frac{\text{digestible NDF}}{\text{NDF intake}} \times 100$

[5] DE: Digestible energy $= \frac{\text{digestible energy intake}}{\text{gross energy intake}} \times 100$

[6] Dairy efficiency $= \frac{\text{Milk yield } \left(\frac{\text{kg}}{\text{d}}\right)}{\text{DMI } \left(\frac{\text{kg}}{\text{d}}\right)}$

[abc] Means in the same row with different superscripts are statistically different at $P < 0.05$

Table 3. Correlation of methane emission (g/d), yield (g/kg DMI), and intensity (g/kg milk yield) in response to diet chemical composition and total tract digestibility, DMI and lactation performance

	CH_4, g/d				CH_4 yield, g/kg DMI				CH_4 intensity, g/kg milk yield			
	n	Intercept (SE)	Slope (SE)	*P*-value	n	Intercept (SE)	Slope (SE)	*P*-value	n	Intercept (SE)	Slope (SE)	*P*-value
Diet composition, % DM												
OM	92	-671 (245)	11.6 (2.65)	<0.01	145	-30.0 (13.9)	0.55 (0.15)	<0.01	137	36.7 (21.3)	-0.22 (0.23)	0.34
NDF	176	542 (21.4)	-3.71 (0.50)	<0.01	164	14.4 (1.68)	0.18 (0.04)	<0.01	158	5.69 (1.84)	0.27 (0.05)	<0.01
Starch	112	431 (14.6)	-0.68 (0.38)	0.07	111	22.7 (0.62)	-0.12 (0.02)	<0.01	106	16.9 (0.61)	-0.11 (0.02)	<0.01
CP	178	422 (23.2)	-1.22 (1.19)	0.31	166	27.3 (0.78)	-0.36 (0.04)	<0.01	158	15.8 (1.91)	0.01 (0.11)	0.92
Total tract digestibility, %												
OMD[1]	97	546 (88.6)	-1.80 (1.17)	0.13	90	32.2 (6.49)	-0.15 (0.09)	0.09	92	29.2 (7.99)	-0.17 (0.11)	0.11
NDFD[2]	74	429 (30.5)	-0.14 (0.43)	0.74	73	17.7 (1.41)	0.05 (0.02)	0.02	70	15.1 (1.36)	-0.005 (0.02)	0.83
Production performance, kg/d												
Milk yield	183	257 (23.9)	5.41 (0.82)	<0.01	173	29.6 (1.30)	-0.32 (0.05)	<0.01	-	-	-	-
DMI	194	127 (25.4)	14.1 (1.25)	<0.01	-	-	-	-	176	27.9 (2.09)	-0.61 (0.11)	<0.01
Milk composition, %												
Fat	174	408 (52.1)	-1.56 (11.7)	0.89	165	3.88 (2.63)	3.96 (0.60)	<0.01	167	1.02 (3.14)	3.51 (0.71)	<0.01
Protein	174	515 (82.1)	-33.3 (23.9)	0.17	165	25.2 (4.62)	-1.23 (1.35)	0.37	167	4.54 (5.33)	3.42 (1.56)	0.03

[1] OMD: $\text{Total tract OM digestibility} = \frac{\text{digestible OM}}{\text{OM intake}} \times 100$

[2] NDFD: $\text{Total tract NDF digestibility} = \frac{\text{digestible NDF}}{\text{NDF intake}} \times 100$

Table 4. Correlation of methane emission (g/d), yield (g/kg DMI), and intensity (g/kg milk yield) in response to intake, lactation performance and diets characteristics of cows fed diets with different forage basis (CS - corn silage; GS - grass silage; GF - green forage)

	CS				GS				GF			
	n	Intercept (SE)	Slope (SE)	*P*-value	n	Intercept (SE)	Slope (SE)	*P*-value	n	Intercept (SE)	Slope (SE)	*P*-value
CH_4, g/d												
Milk yield, kg/d	52	411 (59.5)	0.70 (1.84)	0.71	72	328 (30.5)	3.28 (1.12)	<0.01	52	310 (37.3)	1.97 (1.54)	0.21
DMI, kg/d	56	350 (85.2)	3.78 (3.81)	0.33	72	203 (35.9)	11.2 (1.88)	<0.01	59	45 (40.8)	18.6 (2.38)	<0.01
NDF, %DM	53	461 (73.8)	-0.77 (2.06)	0.71	61	463 (23.7)	-1.25 (0.54)	0.02	55	511 (34.3)	-3.86 (0.73)	<0.01
Starch, %DM	39	501 (40.1)	-3.04 (1.41)	0.04	53	413 (14.0)	-0.05 (0.52)	0.92	13	422 (30.8)	-3.85 (1.04)	<0.01
NDFD[1], %NDF	24	343 (56.8)	2.20 (0.97)	0.04	36	378 (46.1)	0.26 (0.64)	0.69	7	210 (64.1)	1.90 (0.68)	0.049
CH_4 yield, g/kg DMI												
NDF; %DM	48	19.7 (3.88)	-0.01 (0.11)	0.93	60	17.9 (1.68)	0.11 (0.04)	0.01	49	10.3 (4.36)	0.27 (0.10)	0.01
Starch, %DM	39	23.6 (2.00)	-0.19 (0.08)	0.03	52	22.5 (0.69)	-0.03 (0.03)	0.36	13	22.9 (1.28)	-0.22 (0.07)	0.02
CP, %DM	49	16.5 (6.35)	0.18 (0.39)	0.66	60	24.7 (1.14)	-0.15 (0.06)	0.02	49	30.4 (1.08)	-0.44 (0.04)	<0.01
NDFD[1], %NDF	24	11.3 (2.16)	0.18 (0.04)	<0.01	35	24.3 (2.80)	-0.03 (0.04)	0.44	7	11.0 (3.28)	0.12 (0.04)	0.049
CH_4 intensity, g/kg milk yield												
NDF, %DM	46	19.3 (2.91)	-0.17 (0.08)	0.06	57	10.2 (1.65)	0.15 (0.04)	<0.01	48	-1.32 (4.75)	0.46 (0.10)	<0.01
Starch, %DM	38	15.1 (1.63)	-0.05 (0.06)	0.40	49	16.6 (0.80)	-0.05 (0.04)	0.23	13	17.9 (1.16)	-0.25 (0.08)	0.01
NDFD[1], %NDF	23	10.6 (2.03)	0.07 (0.04)	0.10	33	21.8 (2.69)	-0.09 (0.04)	0.03	7	19.4 (4.32)	-0.05 (0.05)	0.40

[1] NDFD: $\text{Total tract NDF digestibility} = \frac{\text{digestible NDF}}{\text{NDF intake}} \times 100$

Table 5. Prediction equations of methane produced by cows fed diets with different forage basis (estimates(±S.E.))

Item	n	Model	RMSE	AICc
CH_4, g/d	68	$-65.3(\pm 63.7) + 11.6(\pm 1.67) \times \mathrm{DMI} - 4.47(\pm 1.09) \times \mathrm{CP} - 0.86(\pm 0.33) \times \mathrm{Starch} + 2.62(\pm 0.78) \times \mathrm{OMD}^1 + 30.8(\pm 9.45) \times \mathrm{Milk\ Fat}$	11.2	597
CH_4 yield, g/kg DMI	84	$6.16(\pm 3.89) - 0.36(\pm 0.03) \times \mathrm{CP} + 0.12(\pm 0.05) \times \mathrm{OMD}^1 + 3.77(\pm 0.56) \times \mathrm{Milk\ Fat} - 3.94(\pm 1.07) \times \mathrm{Milk\ Fat\ Yield}$	1.68	320
CH_4 intensity, g/kg milk yield	130	$-55.5(\pm 20.1) - 0.37(\pm 0.13) \times \mathrm{DMI} + 0.18(\pm 0.05) \times \mathrm{Forage}^2 - 0.10(\pm 0.04) \times \mathrm{Inclusion}^3 + 0.48(\pm 0.21) \times \mathrm{OM} + 0.14(\pm 0.06) \times \mathrm{NDF} + 1.98(\pm 0.86) \times \mathrm{Milk\ Fat} + 4.34(\pm 1.66) \times \mathrm{Milk\ Protein}$	1.56	629

[1] $\mathrm{OMD{:}\ Total\ tract\ OM\ digestibility} = \frac{\mathrm{digestible\ OM}}{\mathrm{OM\ intake}} \times 100$

[2] Forage: forage DM on total diet DM

[3] Inclusion: inclusion (%DM) of the main forage of the diet

Supplementary materials

Supplementary Table 1. Published papers included in the present meta-analysis

Number	Author	Year	Journal
1	Arndt et al.	2015	J. Dairy Sci. 98:418–430
2	Benchaar et al.	2014	J. Dairy Sci. 97:961-974
3	Børsting	2020	J. Dairy Sci. 103:1448–1462
4	Cabezas-Garcia et al.	2017	J. Dairy Sci. 100:5228–5240
5	Carmona-Flores et al.	2020	Animals 10:1301
6	Civiero et al.	2021	J. Dairy Sci. 104 IN PRESS
7	Colombini et al.	2015	Agricultural Systems 136 :106–113
8	Coppa et al.	2020	Methods IN PRESS
9	Dall-Orsoletta et al.	2016	J. Dairy Sci. 99:4374–4383
10	De Boever et al.	2016	J Anim Physiol Anim Nutr. 101:246–256
11	Dini et al.	2012	Animals 2:288-300
12	Dorich et al.	2015	J. Dairy Sci. 98:2676–2681
13	Ellis et al.	2016	J. Dairy Sci. 99:7159–7174
14	Enriquez-Hidalgo et al.	2014	J. Dairy Sci. 97:1400–1412
15	Enriquez-Hidalgo et al.	2020	Frontiers in Veterinary Science 7:935
16	Ferris et al.	2020	Agricultural and Food Science 29(2):130-138
17	Fischer et al.	2019	J. Dairy Sci. 103:4408–4422
18	Gidlund et al.	2017	Livest Sci. 197:73–81
19	Gidlund et al.	2015	J. Dairy Sci. 98:8093–8106
20	Gislon et al.	2020	J. Dairy Sci. 103:8048–8061
21	Günal et al.	2018	Animal Production Science 59(3):499-505
22	Hammond et al.	2016	J. Dairy Sci. 99:7904–7917
23	Harper et al.	2018	J. Dairy Sci. 101:5006–5019
24	Harper et al.	2017	J. Dairy Sci. 100:6151–6163
25	Hart et al.	2015	Animal 9(6):983–991
26	Hassanat et al.	2017	J. Dairy Sci. 100:2625–2636
27	Hassanat et al.	2013	J. Dairy Sci. 96:4553–4567
28	Hassanat et al.	2014	J. Dairy Sci. 97:6463–6474
29	Hatew et al.	2016	J. Dairy Sci. 99:354–368
30	Hatew et al.	2015	J. Dairy Sci. 98:486–499
31	Hynes et al.	2016	J. Dairy Sci. 99:8858–8866
32	Jiao et al.	2014	J. Dairy Sci. 97:1–11
33	Jonker et al.	2019	Animal Production Science 59(6):1063-1069
34	Jonker et al.	2017	Animal Production Science 57(4):643-648
35	Judy et al.	2018	J. Dairy Sci. 101:10899–10910
36	Karlsson et al.	2019	J. Dairy Sci. 102:7927–7935
37	Kidane et al.	2018	Animal Science 68(2):87-102
38	Kidane et al.	2018	J. Anim. Sci. 96:3967–3982
39	Lettat et al.	2013	J. Dairy Sci. 96:5237–5248
40	Liu et al.	2012	J. Environ. Qual. 41(4):1228-1237
41	Moate et al.	2020	J. Dairy Sci. 103:2373–2386
42	Muñoz et al.	2016	J. Dairy Sci. 99:7945–7955
43	Muñoz et al.	2012	J. Dairy Sci. 95:3139–3148

44	Olijhoek et al.	2018	J. Dairy Sci. 101:9926–9940
45	O'Neill et al.	2012	J. Dairy Sci. 95:6582–6590
46	O'Neill et al.	2011	J. Dairy Sci. 94:1941–1951
47	Pang et al.	2018	J. Dairy Sci. 101:7133–7145
48	Pirondini et al.	2015	J. Dairy Sci. 98:357–372
49	Uddin et al.	2020	J. Dairy Sci. 103:6087–6099
50	Van Gastelen et al.	2015	J. Dairy Sci. 98:1915–1927
51	Van Wyngaard et al.	2018	J. Dairy Sci. 101:9954–9966
52	Waghorn et al.	2016	Animal Production Science 56:252–257
53	Warner et al.	2017	J. Anim. Sci. 95:3687–3699
54	Warner et al.	2016	Animal 10(1):34–43
55	Warner et al.	2015	J. Dairy Sci. 98:3383–3393
56	Williams et al.	2016	Animal Production Science 56:304–311
57	Wilson et al.	2020	Journal of Animal Science 98(7) 1–13
58	Wims et al.	2010	J. Dairy Sci. 95:6582–6590

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5. Experiment 3

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Evaluation of IPCC model to predict enteric methane emission on lactating cows fed typical Mediterranean diets

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INTERPRETATIVE SUMMARY

The computation of anthropic greenhouse gases is crucial for decision-making purposes, evaluation of mitigation strategies, and the trend of GHG emissions over time. The IPCC (Intergovernmental Panel on Climate Change) provides guidelines for quantifying GHG, including enteric CH_4 from dairy cows. However, these guidelines might not represent some country-specific situations related to peculiar diets. Therefore, this study proposed new coefficients (methane conversion factor = 5.58%, and digestible energy = 69.4% for silage-based diets or 64.7% for hay-based diets) specific for the diets typical of the Mediterranean region in order to improve the prediction performances of the model from IPCC which, overall, predicted satisfactorily enteric methane emission.

ABSTRACT

This paper aimed to evaluate IPCC (Intergovernmental Panel on Climate Change) Tier 2 to predict enteric CH_4 emission from cows fed Mediterranean diets. The focus was on the CH_4 conversion factor (Y_m) and digestible energy of the diet (DE) as predictors in the model. A dataset was built using individual observations derived from 3 in vivo studies conducted in Italy on lactating cows housed in respiratory chambers. Five models following the Tier 2 approach were evaluated: 1) average values of Y_m (6.5%) and DE (70%), from IPCC (2006) (06); 2) average values of Y_m (5.7%) and DE (70.0%) from, IPCC (2019) (1YM); 3) Y_m = 5.7% and DE measured in vivo (1YMIV); 4) Y_m = 5.7 or 6.1%, depending on dietary NDF, and DE = 70% (2YM); 5) Y_m = 5.7 or 6.1%, and DE measured in vivo (2YMIV). A Tier 2 model for Mediterranean diets (MED) was obtained from the Italian dataset (Y_m= 5.58%; DE= 69.4% for silage-based diets and 64.7% for hay-based diets) and validated on an independent dataset of cows fed a Mediterranean diet.

On average, the in vivo DE was 69.0%, but the diet based on hays had a lower value (64.8%, $P < 0.05$) than the silage-based diets (69.9%, on average). The models 1YMIV, 2YM, and 2YMIV gave predicted values of CH_4 in g/d not different than the observed ones (on average 385 vs. 388 g/d in vivo, $P > 0.05$). Instead, the 06 model over-predicted significantly ($P < 0.05$) CH_4 emission (428 g/d, $\mu = -1.05$). The most precise models (R = 0.630) were the two considering average values of Y_m and DE from IPCC (2006, 2019) (06 and 1YM). Overall, the best performance was for 1YM (concordance correlation coefficient (CCC) = 0.579 and RMPSE = 9.10%). The inclusion of in vivo DE values only improved accuracy (bias correction factor (Cb) = 0.992, on average), compared to models with DE = 70% (Cb = 0.832).

The performances on the external dataset were lower but similar for MED and 1YM (RMPSE = 28.9%). In particular, precision was lower for the prediction of CH_4 emission from cows fed a diet based on fresh herbages (R = 0.277, on average), due to high over-prediction ($\mu = -0.452$), compared to a diet based on corn silage and alfalfa hay which showed high precision (R = 0.857, on average).

The results of this study showed that the average values proposed by IPCC (2019) could adequately predict CH_4 emission from cows fed typical Mediterranean diets. However, the prediction was improved including data measured in vivo, and thus the use of factors specific for Mediterranean diets is advised, even if further studies are required to investigate CH_4 emission from cows fed diets based on hays.

Key words: Tier 2, Italy, digestible energy, hay-based diets

INTRODUCTION

In 2019, the European Commission set the European Green Deal, following the EU commitment defined in the Paris agreement of 2015, to become climate-neutral by 2050 and decrease greenhouse gas emissions (**GHG**) by 55% by 2030 compared to the 1990 level. According to the World Resources Institute (2020), livestock sector and manure management contributes for the 5.8% of human-caused GHG emissions. In particular, considering CH_4 emissions only, enteric fermentation and manure management represented the 32% of anthropic CH_4 emissions (Global Methane Assessment, 2021).

In order to mitigate this environmental impact, it is important to predict the amount of enteric CH_4 emitted in relationship to a specific production system (Niu et al., 2018). However, prediction models give an estimate of the values, not a measure of a phenomena; so it is important to determine whether this prediction is valid and sound and the model accomplished what was expected of it (Tedeschi, 2006). The IPCC

(Intergovernmental Panel on Climate Change) guidelines for GHG inventories were born to provide internationally agreed methodologies to estimate GHG to report to United Nations Framework Convention on Climate Change (IPCC, 2006a). These estimations are conducted according to 3 levels (Tier) of methodological complexity. The latest version of these guidelines (IPCC, 2019b) was published to refine the previous version (IPCC, 2006b). The aim was to provide supplementary methodologies, updated default values based on the latest scientific studies, alternative or updated guidance, and clarification. Regarding enteric CH_4 emission from ruminants, Tier 1 emission factors have been updated, and the conversion factor of gross energy intake (**GEI**) into enteric CH_4 energy (**Y_m**) can be chosen according to the level of productivity and diet characteristic (IPCC, 2019b). In Tier 2 of IPCC (2019a), great importance relies on GEI (based on Net Energy requirements) and Y_m. The criteria to choose the value of Y_m are milk production, NDF concentration, and digestible energy (**DE**; defined as % of GEI) of the diet, highlighting the great importance of diet composition and ingredients in affecting CH_4 production, as also discussed in the recent review of Beauchemin et al. (2020). Considering the whole dataset, IPCC (2019a) suggests a value of Y_m equal to 5.7% for high-yielding cows (> 8500 kg milk/head*year), associated with a diet with DE ≥ 70% and NDF ≤ 35% of diet DM. Moreover, IPCC proposed reference values specifically for Europe: the Y_m value for high producing cows in Europe is 6.1%, with mean dietary NDF equal to 37%, while the value for DE is not specified. The Y_m value suggested for European cows is close to the one (6.0%) of the IPCC dataset associated with diets with NDF > 35% and DE > 70% of GEI. However, for this production level, these values of NDF and DE do not feature the diets of the Mediterranean region. Indeed, it has to be considered that the Y_m values in IPCC (2019a) were based on the dataset of Niu et al. (2018), which includes 154 studies, 82 of them conducted in European research institutes and 64 in the USA. None of these EU studies was conducted in the Southern European countries (38 in the United Kingdom, 23 in Scandinavian countries, 9 in Switzerland, 5 in the Netherland, 4 in Belgium, 1 each in Ireland, Germany, and France). The Y_m value is affected by the type of diet. In this regard, the US diets (based on corn and alfalfa silages, according to Arndt et al., 2015), and the North Europe diets (based on ryegrass and corn silages, according to March et al., 2014) are different from the ones of the Mediterranean region of Europe widely based on corn silage and alfalfa and grass hays (Gislon et al., 2020a). In this study, a survey of 171 Italian Holstein dairy herds showed that in Northern Italy, the majority of farms (> 90%) used corn silage as the main ingredients for lactating cow TMR, with an average NDF concentration of the diets of 33.9% (SD = 3.44). Moreover, alfalfa and meadow hays were used in more than 50% of farms for lactating cow TMR. Part of the reason for the popularity of dry forages in the area is the great importance played by Parmigiano Reggiano cheese in the Italian agri-food sector for which production the use of silage is banned. However, diets based on hay have significant differences compared to diets based on silage; one of the most important is the different DMI which is strongly correlated to methane production (Cavallini et al., 2021). A study conducted in Switzerland (Klevenhusen et al., 2011) tested a diet based solely on hay as a forage source (52.9 % dietary NDF on diet DM and 66% DE), finding a value of Y_m equal to 7.9%; however, the experimental diet was very different from typical Po plain region diets.

For these reasons, IPCC (2019a) encourages the development of country-specific Y_m factors for more accurate estimation. Therefore, the present study aimed to compare the CH_4 emissions estimated with IPCC (2019a) and IPCC (2006b) with the values obtained in vivo in respiratory chambers to develop possible improvements to IPCC parameters for a better prediction of CH_4 emission from lactating cows fed Mediterranean diets.

MATERIAL AND METHODS

Dataset

A dataset of 66 individual observations was created to evaluate the performance of different models in predicting CH_4 emissions from Italian dairy cows. This dataset included individual cow observations of three in vivo studies: Gislon et al. (2020b), Pirondini et al. (2015), and Colombini et al. (2015), with 32, 16, and 18 observations, respectively. These experiments were carried out at the Università degli Studi di Milano "Cascina Baciocca" Research Center, at Cornaredo (Milan, Italy; N 45° 30', E 9° 1'). The climate of the region is relatively cool, mid-latitude version of the Humid subtropical climate (Köppen climate classification Cfa). Methane emission was measured through individual open-circuit respiration chambers. Only lactating Italian Friesian cows were used, arranged in Latin square design. The diets fed in each experiment were defined according to the main forages included as follows. Gislon et al. (2020b) investigated four diets with different roughages types: a diet based on grass and alfalfa silages (**GAS**), one based on grass and alfalfa hay (**HAY**), one on wheat silage (**WS**), and one on corn silage (**CS1**). Pirondini et al. (2015) fed four diets based on corn silage and different NDF and starch contents (**CS2**). However, two diets contained fish oil supplementation to abate CH_4 emission, thus were not considered. Colombini et al. (2015) investigated a diet based on corn silage (**CS3**), and two diets based on sorghum forage and grain silages (**SS**) (with forage sorghum being taller, leafier and grain sorghum producing more grain; Bean et al., 2013). Main diets characteristics and animal responses of the dietary treatments used in the analysis are reported in Table 1, averaging these variables for the two CS2 diets in Pirondini et al. (2015) and the two SS diets in Colombini et al. (2015).

Definition of the models

Five predicting models were defined (Table 2). One was based on IPCC (2006b) Tier 2 (IPCC Table 10.12), considering Y_m = 6.5% and DE = 70% (**06**). Other two models were based on IPCC (2019a) Tier 2 considering the entire dataset (Table 10.12) for high-production cows: one used the average Y_m value (5.7%) and DE = 70% (**1YM**); one Y_m = 5.7% and DE measured in vivo (**1YMIV**). Other two models were based on IPCC (2019a) Tier 2 considering either the value of Y_m suggested for the whole dataset (5.7%), for diets with NDF ≤ 35%, or the value of Y_m suggested for Europe (6.1%,), for diets with NDF > 35%, according to what reported in IPCC Table 10B.1. For this latter model, the value of DE was set either at 70% (**2YM**), the average value reported in IPCC (2019a), or the in vivo value was used (**2YMIV**). The value of in vivo DE used in each of the equations is reported in Table 2; DE value of 64.8% was used for the hay diet while the average value (69.9%) of the other Italian diets was used for silage diets.
Finally, new values of Y_m and DE were obtained, basing on the results of the three in vivo experiments of the present dataset. An IPCC Tier 2 model for cows fed typical Mediterranean diets was proposed using these values of Y_m and DE (**MED**) and it was validated on an independent dataset as described later.

Models evaluation

The predictive models for CH_4 emission were evaluated considering the following statistics. The square root of the mean square prediction error (**RMSPE**) was calculated as follows:

$$\mathrm{RMSPE} = \frac{\sqrt{\left(\frac{1}{n} \times \sum_{i=1}^{n}(O_i - P_i)^2\right)}}{\frac{1}{n} \times \sum_{i=1}^{n} O_i} \times 100$$

where n is the number of observations, O_i is the i^{th} observed value and P_i is the i^{th} predicted value.

The RMSPE was decomposed into error due to overall bias (**ECT**), error due to deviation of the regression slope from unity (**ER**), and error due to the disturbance (random error) (**ED**), according to Bibby and Toutenburg (1977). The ECT, ER, and ED were calculated as below:

$$\mathrm{ECT} = (M_P - M_O)^2$$

$$\mathrm{ER} = (SD_P - R \times SD_O)^2$$

$$\mathrm{ED} = (1 - R^2) \times {SD_O}^2$$

where SD_O and SD_P are the standard deviations of observed and predicted values, respectively, M_O and M_P are the means of observed and predicted values, respectively, and **R** is the Pearson correlation coefficient (a measure of precision). The R ranges from 0 to 1, with 1 indicating the perfect fit (Kebreab et al., 2008).

The analysis of concordance correlation coefficient (**CCC**), where higher CCC indicates a better prediction of the observed values, was conducted according to Lin (1989). The CCC was calculated as follows:

$$\mathrm{CCC} = \mathrm{R} \times \mathrm{Cb}$$

where **Cb** is a bias correction factor (a measure of accuracy). The Cb ranges from 0 to 1; when Cb = 1, there is no deviation from the regression line Y = x (Tedeschi, 2006). Thus, when CCC = 1, there is perfect agreement between the two variables.

The Cb was calculated as below:

$$\mathrm{Cb} = 2/(\mathrm{V} + 1/\mathrm{V} + \mu^2)$$

where **V** is a measure of scale shift (i.e., the change in standard deviation between predicted and observed values), and **μ** is a measure of location shift (i.e., under-prediction with a positive value and over-prediction with a negative value). The V and μ were calculated as below:

$$\mathrm{V} = \mathrm{SD_O}/\mathrm{SD_P}$$

$$\mu = (\mathrm{M_O} - \mathrm{M_P})/(\mathrm{SD_O} \times \mathrm{SD_P})^{\frac{1}{2}}$$

When μ is negative (minimum value = -1) it indicates over-prediction; when μ is positive (maximum value = 1), it indicates under-prediction (Kebreab et al., 2008). The perfect score is 0.

Cross-validation

The models MED and 1YM, defined above, were validated using an external dataset based on single cow observations from the study of Enriquez-Hidalgo et al. (2020). It was not possible to apply the models 1YMIV and 2YMIV because DE was not measured in the above-mentioned study; 2YM was not applied because the two diets investigated had dietary NDF < 35% of diet DM (32.8%, on average). Methane emissions were estimated with the SF_6 technique, and two breeds of cows were implied (Holstein-Friesian and Montbeliard). The experiment took place in a region with a Mediterranean climate, and one of the diets investigated in this paper (**CS**) was similar to the diets typically fed in Italy, as the forage basis included corn silage (34.2% diet DM) and alfalfa hay (8.4%). The other diet was based on corn silage and a mixture of fresh annual ryegrass and berseem clover (**MIX**). Therefore, the evaluation of the predicting models was carried out as explained above for each diet of Enriquez-Hidalgo et al. (2020). For this dataset, the evaluation of the MED model was also applied.

Statistical analysis

Data regarding DE and Y_m emission were statistically analysed by the proc mixed procedure of SAS 9.4, with the following model:

$Y_{jkl} = \mu + D_j + COW_l(S_k) + \varepsilon_{jkl}$

where Y_{jkl} is the dependent variable; μ is the overall mean; D_j is the diet effect (j = 1, 5); S_k is the random study effect (k = 1, 3); COW_l is the random animal effect, nested within the study (l = 1, 18), and ε_{jkl} is the residual error.

Data regarding CH_4 emission were statistically analysed by the proc mixed procedure of SAS 9.4, with the following model:

$Y_{ijkl} = \mu + M_i + D_j + M_i \times D_j + COW_l(S_k) + \varepsilon_{ijkl}$

where Y_{ijkl} is the dependent variable; μ is the overall mean; M_i is the model effect (i=1, 6), including respiration chamber; D_j is the diet effect (j = 1, 5); $M_i \times D_j$ is the interaction between the two factors; S_k is the random study effect (k = 1, 3); COW_l is the random animal effect nested within the study (l = 1, 18), and ε_{ijkl} is the residual error.

Least squares means estimates are reported. For all statistical analyses, significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

RESULTS

Digestible energy and Y_m values of cows fed Mediterranean diets with a different forage basis

Digestible energy and Y_m of cows fed Mediterranean diets are shown in Table 3. The mean DE was affected by the diet ($P < 0.001$). The DE (%) was significantly lower ($P < 0.05$) for the HAY diet compared to the others, except for CS3. The DE was significantly higher ($P < 0.001$) for the CS1 diet compared to the others, except for GAS. Intermediate values of DE were detected for GAS, WS, CS2, CS3, and SS diets.
The mean Y_m (%) was also affected by the diet ($P = 0.007$). The CS3 diet had a lower value than the other diets except for CS2 ($P < 0.05$), which was intermediate.
Based on these results, Mediterranean coefficients of Y_m and DE for a Mediterranean model (MED) were proposed as follows: Y_m= 5.58% (average of all Y_m values); DE= 69.9% and 64.8% respectively for silage and hay diets.

In vivo enteric methane production and IPCC estimates of cows fed Mediterranean diets

Five predictive models for estimating enteric methane emissions related to typical Mediterranean diets were applied from IPCC (2006b) and IPCC (2019a) Tier 2 equations. Results of enteric CH_4 in terms of daily, yield (g/DMI), and intensity (g/milk yield) obtained using these predicting models are presented in Table 4. The predicted values were also compared with measured in vivo data obtained in respiration chambers.
The method affected all the variables investigated ($P < 0.001$). Thus, 06 over-estimated the emissions compared to the in vivo values for all the variables. The daily methane emission was, on average, under-estimated when 1YM was applied. Instead, 1YMIV, 2YM, and 2YMIV were not different from in vivo values. However, the accuracy and precision of the evaluated equations were different (Table 5). The RMSPE (%) was similar among models (on average 10.6%). The 06 was the least accurate model, followed by the values obtained with 1YM; however, this latter model was the most precise as shown by the lower ER. The use of in vivo DE value increased the ER and decreased the ED. The prediction with 2YMIV resulted in the highest ER and the lowest R between predicted and observed values. The overall evaluation of equations by CCC showed the highest rank for the model 1YM followed by 1YMIV. Two equations (06 and 2YMIV) over-estimated the CH_4 prediction.

Validation of IPCC model and IPCC correction factors for Mediterranean diets on an independent dataset

The cross-validation results on the independent dataset applying the 1YM and the MED equations are in Table 6. The RMSPE (on average 28.9%) was slightly higher than the results on the Italian dataset and higher for CS than MIX. However, the results showed a better prediction for 1YM and MED on CS than MIX diets. Particularly, the ECT was higher for the prediction on MIX (7.74% on average) than CS (0.278% on average), and it was lower for the MED model than the 1YM model. The ED value was higher for MIX (91.8% on average) than CS (46.9% on average) diets; however, the R values were very low for the prediction of MIX diet (on average R = 0.277%) compared to CS (on average 0.857%). Overall, the CCC was very low for MIX (on average 0.171) and higher for CS (on average 0.489) using both models. The predicted results were higher than the in vivo values but differed between diets with a higher over-prediction for the MIX diet than the CS diet. The MED model resulted in lower µ values than $1Y_m$ for both diets.

DISCUSSION

Methane conversion factor and digestible energy of cows fed Mediterranean (Po plain) diets

The present study aimed to evaluate the IPCC (2019a) Tier 2 method and propose country-specific values of Y_m and DE to predict enteric CH_4 emissions from lactating cows reared in an intensive dairy system of the Mediterranean climate. The IPCC (2019a) dataset is based on Niu et al. (2018) study and, for Europe, most studies were conducted in countries with feeding systems and environmental conditions different from that of the Mediterranean climate type of the Po plain; hence some differences can be expected. For example, the average milk production reported by Niu et al. (2018) for the European dataset is 26.4 kg/d; a value lower than that (30.0 kg/d) reported by Pirondini et al. (2012) or by Gislon et al. (2020a) (29.8 kg/d) for dairy cows in the Po plain. Moreover, the IPPC (2019a) Tier 2 for the European dataset (milk yield > 8500 kg) assumes a Y_m value of 6.1%. This value is higher than the average value (5.58) observed in the Italian dataset. A meta-analysis study (Rota Graziosi et al., 2021) underlined that in Europe, the most investigated forages were grass silage, followed by corn silage and green forage. Diets based on grass silage are expected to have higher NDFD and Y_m values than diets based on corn silage; for example, Hart et al. (2015) reported a higher CH_4 production, expressed relative to total GEI, for cows offered grass silage compared with cows offered corn silage. Similarly, Benchaar et al. (2014) and Hassanat et al. (2013) showed that increasing the proportion of corn silage at the expense of alfalfa and barley silage reduces CH_4 emissions on a GE basis. The present study results confirmed a higher CH_4 energy loss for GAS, WS, and HAY diets than 2 out of 3 CS diets of the dataset. Moreover, the average Y_m for CS diets of Po Plain was 5.38 %, and this value is slightly higher than the Y_m value (5.2%) derived from IPCC North America studies. The difference is partially due to Po plain diet composition. While in the Po plain, corn silage is used in combination with hays rather than silages (Gislon et al., 2020a), in North America the main forages of the computed national US average dairy cow ration are corn silage and alfalfa, mainly as silage rather than hay (Martin et al., 2017). The higher use of hays in the Po plain TMR can partially explain the higher Y_m than North America. For example, Martin et al. (2016) reported a value of CH_4 energy loss (% GEI) of 7.1% for cows fed a diet with about 50% of hay (from natural grassland) on total DM and 5.0% for cows fed a diet based on corn silage (55.0% total DM) and a low amount of hay (from natural grassland) (4.5% total DM) as TMR forage basis. Liu and Shi (2019) reported, these results confirmed the need to identify research gaps in estimating Y_m values in literature, quantifying the uncertainties, and highlighting the main sources of variation. Critical differences were already identified by IPCC (2019a) between the North American and the European feeding and production systems. The strongest contrasting factor was the proportion of NDF in the diets of the two regions. As reported in Niu et al. (2018), the EU diets contain more forage and have a greater digestibility of NDF than more concentrate-based US diets; this difference can affect the emission of enteric CH_4.

For example, digestibility data should be based on measured values for the dominant feeds or forages being consumed by livestock. Due to significant variation, digestibility values should be obtained from local scientific data wherever possible. As confirmed by the present study results, there was a wide variability for diet DE depending on the main dietary forage, with the lowest value for HAY. Several studies (Gislon et al., 2020b; Beauchemin et al., 1997; Broderick, 1995) reported a higher DMI for cows fed hay-based diets than silage diets, and increased intake may increase passage rate and shorten retention time in the rumen, thus decreasing the energy digestibility.

For this reason, two different DE values were proposed in this study: 64.8 and 69.9% for hay and silage-based diets, respectively. There is scarce information about the DE measured in vivo of cows fed hay diets: among these, Klevenhusen et al. (2011) reported 65.3% DE in lactating cows, while Hironaka et al. (1996) reported values of 63.7 and 59.4% DE, respectively, for a first and a second cut of alfalfa hay in steers. These values are comparable to the Italian dataset for HAY diet and underlined the need of using a different Y_m for hay rich diets.

Validation of IPCC model on the Mediterranean dataset

Many countries have set targets for reducing GHG emissions, and the quantification of emissions is essential to evaluate mitigation strategies and the trend of GHG emissions over time. Therefore, national emissions inventory reports have become the main instrument for reporting emissions.

Several empiric or mechanistic models can be used to estimate emissions and the advantage of the empiric models is that they require fewer variables and are generally easier to use (Niu et al., 2018). Among the empirical models, the indication provided by IPCC is used globally. However, an evaluation of the IPCC models (2006b, 2019a) in terms of precision and accuracy on cows fed Mediterranean diets is lacking. The accuracy and precision of the model used are important in setting achievable reduction targets, and the present study also aimed to evaluate IPCC (2019a) refinement for cows fed Mediterranean diets compared with the previous IPCC (2006b). In the present study, mean and slope bias of predictions and CCC were used for modelling comparison, and since CCC accounts simultaneously for accuracy and precision, it appeared to provide an efficient way of evaluating model overall performance (Ellis et al., 2010).

The previous IPCC version (2006b) showed some critical issues, such as updating the Y_m constant to be revised downwards for cows in North America (Appuhamy et al., 2016). Similarly, for the Mediterranean dataset, the average CH_4 daily production calculated with 06 (Y_m = 6.5%) was higher than the in vivo or the IPCC (2019a) estimates, and resulted in a lower CCC value than IPCC (2019a) predictions. To the best of our knowledge, only one study (Benaouda et al., 2020) applied the IPCC (2019a) refinement to estimate enteric CH_4 by cattle in Latin America, and the results showed that, compared with IPCC Tier 2 (2006b), the new factors of IPCC (2019a) made a substantial improvement in the prediction of enteric CH_4. Similarly, as Amon et al. (2021) reported for Austria, the refinement of the Y_m is expected to have a distinct impact on enteric CH_4 emission, and the new factors will more accurately reflect the actual situation, as observed for the Italian dataset. The results confirmed the importance of the in vivo Y_m value to estimate the CH_4 emission correctly and that different Y_m values could be applied within Europe. For example, a recent study showed that the predicted Y_m ranged from 6.22 to 6.72% for Norway (Niu et al., 2021), whereas in the Netherlands, a Tier 3 approach used a predicted Y_m of 5.88% to 6.07% (Bannink et al., 2011). Similarly, the results of Mangino et al. (2003) confirmed a wide variability for Y_m value for US cows (from 4.8 to 5.8%).

In the present study, the lowest value of RMSPE was found for 1YM, but all the values were very close. As a comparison, in Benaouda et al. (2019), the models for predicting enteric CH_4 from beef cattle had RMSPE > 34%. For this reason, they were considered not satisfactory, and it was suggested to develop other models. In the present study, all the values of RMSPE were < 13%. Thus, all the models tested had a similar fitting with the present dataset, probably because all the models used the same predictors. In the above-mentioned study of Benaouda et al. (2019), 13 models were evaluated on the same dataset, with a combination of different predictors for each model. The RMSPE ranged from 15.6% to 27.4%. The highest ECT was found for 06, confirming the high bias of this model. This bias resulted in an over-prediction (μ), and 06 and 2YMIV were the only models giving a higher value of CH_4 emission compared to the in vivo measurements. The over-prediction of 06 model was also found by Appuhamy et al. (2016) in North America. On the opposite, ECT was lowest for the 2YM model; this model also had the highest value of ED, highlighting that its RMSPE was mainly due to general disturbance and not to a bias. All the models had medium values of CCC, mainly because of medium to large precision (R) since accuracy was high and close to one for all of them (Cb), except for 06. The most precise models were 1YM and 06. The most accurate was 1YMIV, very close to 2YMIV. Thus, DE measured in vivo improved accuracy of the models, as confirmed also considering V: the models that best predicted the in vivo variability (V close to 1) were the ones using DE measured in vivo. Unfortunately, using a value of in vivo DE resulted in a lower precision, especially for 2YMIV. Including data measured in vivo in Italy did not significantly improve the performance of the predicting model but just the accuracy, which in turn resulted in predicted CH_4 not different from in vivo values for 1YMIV and 2YMIV.

Overall, the best model for CCC was 1YM. Regarding μ, the best model was 2YM. Storlien et al. (2014) considered that a value of μ equal to 0.07 was a measure of a negligible under-prediction. In the present study, 1YM and 1YMIV have μ very close to this value, while for 2YM it was even lower.

According to Kaewpila and Sommart (2016), when a model had RMSPE > 16.0%, or CCC < 0.27, this is considered inadequate. None of the models evaluated here fell into this category. For example, for all the models evaluated, the RMSPE was lower than the values (16.2 and 11.5%) reported by Niu et al. (2018) and by Appuhamy et al. (2018) applying IPCC (2006b) Tier 2. In this study, the best model combining RMSPE and CCC was 1YM, despite higher bias than the models including data measured in vivo.

The results confirm that two critical factors can affect the IPCC prediction and the values of accuracy and precision: the knowledge of in vivo DE and the Y_m coefficient. Such default and fixed estimates may not address the variation encountered in commercial production due to cow type (i.e., feed intake, cow productivity), diet composition, and dietary characteristics (Bannink et al., 2011). For this reason, different Y_m values, depending on milk production, dietary NDF, and country, were proposed by IPCC (2019a). Moreover, to improve the prediction, including a DE value determined in vivo should be advisable (Cb was

increased from 0.930 to 0.992, on average, including DE in vivo). Despite this, the estimate applying 2YMIV resulted in the prediction with the lowest CCC, the highest ER, and an over-prediction of the emission.
In the present dataset, three dietary treatments had a dietary NDF content > 35%: however, for these diets, the in vivo Y_m was lower than that proposed by IPCC (6.1%), which explains the over-estimation of the model and its lower performance. On the basis of these results, it is not advisable to use two different Y_m values depending on diet NDF for lactating cows fed Mediterranean diets.

Cross-validation of IPCC (2019) and Mediterannean equation on an independent dataset

The cross-validation of MED and 1YM on an independent dataset (Enriquez-Hidalgo et al., 2020) showed that 1YM and MED resulted in similar predictive performances, but the prediction for cows fed MIX diet was not precise, as shown by the low R value and resulted in a high over-estimation of the emission.

The reasons for the peculiar behaviour of MIX diet, in terms of prediction of CH_4 emission with the models applied may be different. In Enriquez-Hidalgo et al. (2020), Milk production was reduced with MIX (31.9 vs. 36.4 kg/d of CS) despite no changes in DMI and CH_4 emission, probably due to a CP deficiency in MIX (CP concentration was 14.8%). This deficiency could have hampered also the performance of rumen methanogens, reducing CH_4 emission compared to what expected. Furthermore, berseem clover, included in MIX, was expected to have potential in mitigating methanogenesis (Enriquez-Hidalgo et al., 2020). Methane emission and yield were numerically reduced also in vivo with cows fed MIX compared to CS (332 vs. 381 g/d for CH_4 emission, and 13.8 vs. 15.5 g/kg DMI for CH_4 yield). Both 1YM and MED prediction were lower for MIX (366 and 358 g/d, respectively) than for CS (405 and 397 g/d, respectively). Nevertheless, the estimations for MIX over-predict the value of CH_4 measured in vivo (334 g/d considering the average of the single observations; $\mu < 0$). Both 1YM and MED did not perform well with MIX, probably because diets like MIX are poorly represented in both datasets. To confirm this, R was much reduced for MIX (0.277 with both 1YM and MED), compared to CS (0.858 for 1YM and 0.856 for MED) and to the diets of the Italian dataset (R ranging from 0.459 to 0.630).

The RMSPE resulted higher than the values registered in the chambers. Part of the reason for this result could be the method used to measure CH_4 emission (respiration chambers for building the MED model, SF_6 in the dataset for cross-validation). In Enriquez-Hidalgo et al. (2020), the SF_6 technique was modified according to Deighton et al. (2014), a method that reduced the variability of CH_4 yield estimation between cows, obtaining an accuracy similar to respiration chambers. However, respiration chambers remain the gold standard method for measuring CH_4 emission due to their precision and accuracy (Garnsworthy et al., 2019). Though, there was good agreement between the prediction models evaluated and the value of CH_4 emission measured in vivo for CS (405, 397, and 396 g/d for 1YM, MED, and in vivo, respectively), with good values of R, as discussed above. Instead, the accuracy (Cb ranging from 0.565 to 0.621) was higher with the Italian dataset using respiration chambers (Cb ranged from 0.636 to 0.995).

CONCLUSIONS

The present study showed a significant improvement in IPCC 2019 compared to the 2006 version to predict enteric CH_4 emission. On average, the refinement model was also adequate for Mediterranean diets. However, the dataset built was a useful tool to estimate new values for Y_m and DE, focusing on diets poorly represented in the dataset used by IPCC. Some diets widely used in the Mediterranean climate regions, such as hays-based diets, are still poorly investigated by the scientific literature in terms of enteric CH_4 emission. Thus, more studies are required to fill this knowledge gap. The development of a country-specific equation is still advisable, even if the Tier 2 model using average values of Y_m and DE proposed by IPCC (2019) proved adequate for the Italian dataset.

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Table 1. Descriptive statistics of the dataset, divided by study

	Study						
	Gislon et al. (2020b)				Pirondini et al. (2015)	Colombini et al. (2015)	
Item	CS1	GAS	WS	HAY	CS2[1]	CS3	SS[2]
n	8	8	8	8	8	6	12
NDF (% of DM)	32.8	27.1	33.7	36.6	33.0	36.6	36.4
NFC (% of DM)	41.2	44.3	38.6	38.5	43.1	36.1	33.7
DMI (kg/d)	20.3	20.9	20.9	23.4	22.8	20.0	19.1
Milk yield (kg/d)	27.0	27.3	28.2	29.3	27.5	25.4	24.1
CH_4 (g/d)	378	396	396	413	404	332	342

Abbreviations: CS = diet based on corn silage (different number for each experiment); GAS = diet based on grass and alfalfa silage; WS = diet based on wheat silage; HAY = diet based on grass and lucerne hay; SS= diet based on sorghum silage.

[1] Average values of the two diets based on corn silage and without fish oil supplementation in Pirondini et al. (2015).

[2] Average values of the two diets based on sorghum silage in Colombini et al. (2015).

Table 2. Summary of IPCC equations applied in the present study

Model	Code	Diet NDF (% of DM)	Y_m (%)	DE (%)
IPCC (2006b)	06	-	6.5	70
IPCC (2019a)	1YM	-	5.7	70
IPCC (2019a)	1YMIV	-	5.7	64.8 / 69.9[1]
IPCC (2019a)	2YM	<35	5.7	70
		>35	6.1	70
IPCC (2019a)	2YMIV	<35	5.7	69.9[1]
		>35	6.1	64.8[1]

[1] 64.8% for the hay diet, 69.9% for silage diets.

Table 3. Digestible energy (DE) and Y_m determined in cows fed Mediterranean diets with a different forage basis

	Study								
	Gislon et al. (2020b)				Pirondini et al. (2015)	Colombini et al. (2015)			
Item	CS1	GAS	WS	HAY	CS2	CS3	SS	SEM	*P*-value
DE (%)	73.6^{a}	72.6^{ab}	70.3^{bc}	64.8^{e}	68.6^{cd}	65.2^{de}	68.9^{c}	1.30	<0.001
Y_m (%)	5.67^{a}	5.92^{a}	5.82^{a}	5.68^{a}	5.41^{ab}	5.05^{b}	5.52^{a}	0.208	0.007

Abbreviations: GAS = diet based on grass and alfalfa silage; HAY = diet based on hay; WS = diet based on wheat silage; CS = diet based on corn silage (different number for each experiment); SS= diet based on sorghum silage.

[1] Average values of the two diets based on corn silage and without fish oil supplementation in Pirondini et al. (2015).

[2] Average values of the two diets based on sorghum silage in Colombini et al. (2015).

$^{a-e}$Values within a row with different superscripts differ significantly at $P < 0.05$.

Table 4. Methane production (g/d) by lactating cows fed Mediterranean diets measured in the respiratory chamber or calculated with different IPCC equations

	Model							*P*-value	
Item	In vivo	06	1YM	1YMIV	2YM	2YMIV	SEM	Method	Diet
CH_4, g/d	388^{b}	428^{a}	375^{c}	383^{bc}	382^{bc}	390^{b}	12.1	<0.001	<0.001
CH_4, g/kg DMI	18.4^{b}	20.2^{a}	17.7^{c}	18.0^{bc}	18.0^{bc}	18.3^{b}	0.324	<0.001	0.02
CH_4, g/kg milk	14.4^{b}	15.8^{a}	13.9^{c}	14.1b^{c}	14.1b^{c}	14.4^{b}	0.323	<0.001	<0.001

Abbreviations: 06: Y_m = 6.5%, DE = 70%; 1YM: Y_m = 5.7%, DE = 70%; 1YMIV: Y_m = 5.7%, DE = 69.9 / 64.8%; 2YM: Y_m = 5.7 / 6.1%, DE = 70%; 2YMIV: Y_m = 5.7 / 6.1%, DE = 69.9 / 64.8%.

$^{a\text{-}c}$Values within a row with different superscripts differ significantly at $P < 0.05$.

Table 5. Results of RMSPE and CCC analysis for the individual lactating cow database

Model	[1]RMSPE%	[2]ECT%	[3]ER %	[4]ED %	[5]CCC	[6]R	[7]Cb	[8]V	[9]μ
06	12.6	59.7	2.96	38.0	0.400	0.630	0.636	1.18	-1.05
1YM	9.10	9.69	1.88	89.9	0.579	0.630	0.919	1.35	0.088
1YMIV	10.1	0.968	19.2	81.5	0.569	0.572	0.995	1.03	0.091
2YM	9.47	0.06	3.69	97.8	0.508	0.539	0.941	1.42	0.025
2YMIV	11.7	1.74	31.0	68.8	0.454	0.459	0.989	0.947	-0.138

Abbreviations: 06: Y_m = 6.5%, DE = 70%; 1YM: Y_m = 5.7%, DE = 70%; 1YMIV: Y_m = 5.7%, DE = 69.9 / 64.8%; 2YM: Y_m = 5.7 / 6.1%, DE = 70%; 2YMIV: Y_m = 5.7 / 6.1%, DE = 69.9 / 64.8%.

[1] Root mean square prediction error expressed as a percentage of the observed mean.

[2] Error due to bias, as a percent of total RMSPE.

[3] Error due to regression, as a percent of total RMSPE.

[4] Error due to disturbance, as a percent of total RMSPE.

[5] Concordance correlation coefficient, where CCC = R x Cb.

[6] Pearson correlation coefficient.

[7] Bias correction factor.

[8] Scale shift.

[9] Location shift.

Table 6. Results of RMSPE and CCC analysis for independent individual lactating cow database derived from Enriquez-Hidalgo et al. (2020)

Model	In vivo CH_4 (g/d)	Predicted CH_4 (g/d)	[1]RMSPE %	[2]ECT%	[3]ER %	[4]ED %	[5]CCC	[6]R	[7]Cb	[8]V	[9]μ
CS											
1YM	396	405	30.8	0.547	52.3	47.1	0.492	0.858	0.573	3.16	-0.098
MED	396	397	31.5	0.009	53.2	46.7	0.485	0.856	0.565	3.22	-0.013
MIX											
1YM	334	366	26.7	9.70	1.03	90.3	0.169	0.277	0.610	2.64	-0.511
MED	334	358	26.7	5.78	0.904	93.3	0.172	0.277	0.621	2.69	-0.392

Abbreviations: 1YM: Y_m = 5.7%, DE = 70%; MED: Y_m = 5.58%, DE = 69.9 / 64.8%; CS = diet based on corn silage; MIX = diet based on fresh annual ryegrass and berseem clover.

[1] Root mean square prediction error expressed as a percentage of the observed mean.

[2] Error due to bias, as a percent of total RMSPE.

[3] Error due to regression, as a percent of total RMSPE.

[4] Error due to disturbance, as a percent of total RMSPE.

[5] Concordance correlation coefficient, where CCC = R x Cb.

[6] Pearson correlation coefficient.

[7] Bias correction factor.

[8] Scale shift.

[9] Location shift.

6. Experiment 4

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Partial replacement of soybean meal with soybean silage in lactating dairy cows diet: part 1, milk production, digestibility, and N balance

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Partial replacement of soybean meal with whole-plant soybean silage in lactating dairy cows diet: part 1, milk production, digestibility, and N balance

The high reliance of the European livestock sector on imported soybean meal (SBM), especially from South America, poses environmental problems, like greenhouse gas emissions for transportation and land-use change with the loss of carbon stock and biodiversity. Aim of the present study was to evaluate the partial substitution of SBM with whole-plant soybean silage in the diet of dairy cows. Thirty-six lactating Holstein cows were arranged according to a change-over design, with 2 weeks of adaptation and 5 days of sampling per period. A control diet (CON) was based on maize silage and SBM, representing 10.7% of total dry matter (DM). In a soybean silage diet (SBS) 35% (on DM basis) of SBM was replaced by soybean silage The dietary treatment did not affect DM intake, milk production, and dairy efficiency while cows fed SBS resulted in lower milk crude protein (3.43 vs. 3.55%, $P < 0.001$) and higher milk urea (30.5 vs. 28.7 mg/dL, $P = 0.002$), in comparison with CON. Nutrients digestibility was lower for SBS than CON; particularly fibre digestibility was 31.5 vs. 38.8% ($P < 0.001$). The efficiency of nitrogen utilization was higher for CON than SBS (32.7 vs. 31.3%, $P = 0.003$). Soybean silage did not penalize feed intake and milk production. However, to fully exploit this forage, digestibility, and nitrogen utilization efficiency should be improved.

Keywords: legume silage; protein feed; ruminant

Highlights

Soybean silage can substitute one-third of soybean meal in dairy cow diet

- Soybean silage inclusion in the diet did not affect milk yield and DMI
- Soybean silage inclusion in the diet reduced N use efficiency

Introduction

Soybean meal (SBM) is the main protein feed source used in the EU (European Commission 2020). The reasons for its popularity are the high crude protein (CP) concentration (up to 53.8% of the DM, as reported by NRC 2001 for decorticated soybean meal), the optimal amino acid profile, and, in particular, the high content of Lysine (6.29% of total CP; NRC 2001). In the period 2019-2020 in the EU, 29.2 million tonnes of SBM were used as feed, and 97% of this amount was not produced in the EU. The data updated to April 2019 showed that the USA (36%) and Brazil (34%) are the main exporters of SBM to Europe (European Commission 2020). In Italy, SBM is among the most economically convenient protein sources on the market (granariamilano.org). It is by far the most used meal from oilseed, accounting for 76% of total oilseed meals in 2019; regarding the supply, 50% SBM used in Italy in 2019 was imported, and 33.8% was produced locally from imported seeds (ASSALZOO 2020).

In Brazil, significant areas of the Amazon forest and the *cerrado* have been cleared to increase the arable land needed for this crop. This was linked with the problem of land-use change and the related loss of biodiversity and carbon stock (Bickel and Dros 2003), causing a high environmental cost linked to the production of soybean. Transportation represents another source of greenhouse gases related to Brazilian SBM use, not only overseas but also within Brazil, due to the predominance of road transportation (Prudêncio da Silva et al. 2010).

For these reasons, there is a need to reduce the inclusion of imported SBM in dairy cattle diets by finding alternative protein sources (Wilkinson and Young 2020). With this regard, self-produced whole-plant soybean silage could represent an alternative source to SBM. Compared to SBM (NRC 2001), CP and RUP concentrations of soybean silage are lower (CP is around 20% of DM), but this silage is also a source of energy, in the form of NDF (45%) and, mostly, EE (up to 8% at maturity stage R7-8) (Tabacco et al. 2019). Furthermore, from an agronomic and environmental standpoint, on farm cropping of grain legumes, like soybean, provide several benefits (Stagnari et al. 2017; Watson et al. 2017). Compared to lucerne or other perennial legumes, soybean has the advantage that the soil is not occupied over multiple years (Seiter et al. 2004), and for this reason, this crop can be inserted in a flexible rotation with maize silage. Despite the low concentration of water-soluble carbohydrates, and the high content of protein, oil, and ash, which could increase the buffering capacity, soybean can be well preserved as silage (Mustafa and Seguin 2003).

To the best of our knowledge, only a few studies determined the nutritive value of whole-plant soybean silage. Beside the above mentioned work of Mustafa and Seguin (2003), the *in vitro* study of Spanghero et al. (2015) found higher CP, EE and NDF digestibility (NDFD) with advancing plant maturity (i.e. from R4 to R6).

As far as we know, three *in vivo* studies conducted outside Europe and one in an Italian commercial farm tested whole-plant soybean silage in the ration of dairy cows. In Vargas-Bello-Pérez et al. (2008), soybean silage was used in substitution of lucerne silage, while in Ghizzi et al. (2020) and Silva et al. (2021), in substitution of maize silage. In all of these works, DMI was reduced by the inclusion of soybean silage, probably due to lower NDFD leading to lower milk yield in Vargas-Bello-Pérez et al. (2008) and Ghizzi et al. (2020). In Silva et al. (2021), digestibility was not affected by the treatment and thus milk yield was not reduced. In contrast, in the study conducted in Italy (Tabacco et al. 2019), soybean silage partially replaced SBM and cotton seeds, leading to increased DMI but had no effect on fat-corrected milk production. Regarding N balance, Ghizzi et al. (2020) found lower milk protein and Silva et al. (2021) lower N intake for the soybean silage diets.

Low NDFD and N use efficiency appear to be possible weak points of feeding soybean silage to dairy cows. On the other hand, reduction of SBM in the ration of high-yielding dairy cows seems to be feasible and not penalizing for production, as found by Gislon et al. (2020). We hypothesized that a reduction of about 35% of SBM could lead to the same lactation performances of a control diet based on SBM as the main protein source.

Given these considerations, the aim of this study was to evaluate the effects of partial replacement of SBM with self-produced whole-plant soybean silage on DMI, milk yield, digestibility, and nitrogen balance.

Material and Methods

Silage preparation

This experiment was conducted in the experimental farm, Angelo Menozzi, of the University of Milan (Italy), located in Landriano (Pavia, Italy). Soybean (hybrid Buenos, class 1+; Limagrain Italy, Parma, Italy) was sown on June 3rd 2018, in a medium consistency soil. The crop was harvested and chopped to a theoretical length of cut of 1.7 cm, on September 27th 2018, when the dry matter content of the crop was 26.2%, at the R6 stage (as described by Fehr et al. 1971). The silage was stored in silo tube bags for 50 days.

Two days prior to harvest, 5 plots of 1 m^2 were hand-harvested and from each plot a subsample of whole-plants was kept while the other plants were divided into stalks, leaves, pods and seeds.

Lactation trial

Thirty-six Holstein cows (initial DIM = 159 ± 45.0 d, initial BW = 632 ± 75.0 kg, parity = 1.80 ± 0.90) were housed in a free-stall barn with cubicles and with free access to drinking water. Cows were fed once per day at 1000, and the TMR was pushed toward the cows several times per day. Each cow was milked twice per day, at 0900 and 2000 hours.

According to DIM and milk production at the beginning of the experiment, cows were divided into two groups, arranged in a change-over design. Two weeks for adaptation to the experimental diets were followed by five days of sampling collection for each of the two periods of the experiment; after the first period, cows had nine days of transition to the new diet.

The two experimental diets were formulated using the CNCPS model (version 6.5; Cornell University, Ithaca, NY) to meet the nutrient requirements of the lactating cows at the beginning of the experiment. The control diet (CON) included 10.7% SBM on DM (Table 1). The soybean silage diet (SBS) was characterized by the inclusion of 12.4% soybean silage on total ration DM and 6.91% SBM. Maize silage, high moisture maize, and maize meal were included in the two diets in different amounts in order to provide the same concentrations of NDF and starch (respectively 31.5% and 28.6% on average) (Table 2).

Samples collection

Samples of TMRs and the ingredients were collected three times in each experimental period. Spot samples of urine and faeces were collected twice, on day 1 and 5 of each experimental period, seven h after feeding. One aliquot of urine (10 ml) was added with 4.078 N sulphuric acid in the ratio of 20:1 (vol/vol) for the analysis of N concentration, while a second aliquot (10 ml) with 0.072 N sulphuric acid in a ratio of 1:4 (vol/vol), for the analysis of creatinine and purine derivatives (PD) concentrations. At the beginning and at the end of each experimental period, BW was recorded using a digital scale (with 4 weight sensors SB1, from PTM, Brescia, Italy). Individual daily milk production was electronically recorded during the two experimental weeks. A milk sample for every cow was collected on days 1, 3 and 5 of each experimental week in both morning and evening milking, with 2-bromo-2-nitro-1,3-propanediol as a preservative and stored at 4°C before analysis. Milk samples were analysed for protein, fat, lactose, urea, casein, SNF, acetone and BHB content using a Fourier transform infrared (FTIR) analyser (MilkoScan FT6000; Foss Analytical A/S), while somatic cell count was carried out with differential count (Fossomatic^{TM} 7; Foss A/S, Hillerod, Denmark). Fat and protein corrected milk (FPCM) was calculated according to NRC (2001).

Individual DMI estimation

Individual DMI was estimated according to equation 11 of Dórea et al. (2017). This equation considers the ratio of allantoin:creatinine in the urine, BW, and milk yield.

Urine allantoin was measured through the method of Chen and Gomes (1992), using Biochrom Libra S11 Visible Spectrophotometer (Biochrom Ltd., Cambridge, United Kingdom); urines creatinine was measured using ILab Aries (Werfen, Milan, Italy) (Jaffé 1886).

Total urine output was estimated according to Valadares et al. (1999), using creatinine concentration in spot urine sample and considering a daily creatinine excretion rate of 29 g/kg of BW. A correction factor of 0.667 was used to take into account the diurnal variation of creatinine in the spot samples, according to the work of Lee et al. (2019).

Chemical analyses

All samples were stored at −20°C. Before analysis, samples of feeds and faeces were thawed and oven-dried at 55°C until constant weight and ground through a 1-mm screen (Pulverisette 19, Fritsch, Idar-Oberstein, Germany).

Feeds and faeces were analysed for the concentrations of DM, ash, ether extract, starch (AOAC 1995, numbers 945.15, 942.05, 920.29, and 996.11, respectively), NDF corrected for insoluble ash and with the addition of α-amylase (aNDFom; Mertens et al. 2002), ADF and ADL (Van Soest et al. 1991), using the Ankom 200 fiber apparatus (Ankom Technology Corp., Fairport, NY). All samples, including urines, were analysed for CP (N × 6.25) (AOAC 1995, numbers 990.03) using Rapid MAX N Exceed Elementar (Elementar Analysensysteme GmbH, Germany). For the two diets and the two main forages, protein fractions were estimated according to CNCPS method (Sniffen et al. 1992), following the analysis procedure of Licitra et al. (1996). A silage sample was divided into 2 subsamples. The first subsample was extracted for pH determination using a Stomacher blender (Seward Ltd., Worthing, UK) for 4 min in distilled water at a 9:1 water-to-sample material (fresh weight) ratio. The second subsample was extracted using a Stomacher blender for 4 min in 0.05 M sulfuric acid (H_2SO_4) at a 5:1 acid-to-sample material (fresh weight) ratio. A 40-mL aliquot of silage acid extract was filtered with a 0.20-μm syringe filter and used to quantify the fermentation products. Lactic, acetic, propionic, and butyric acids were determined by means of HPLC in the acid extract (Canale et al. 1984). Kjeldahl method was used for the determination of $N\text{-}NH_3$ of the silages.

In vitro analyses

Four in vitro incubation were conducted:

(1) A 48 h incubation using glass syringes to determine the net energy for lactation (NEl) of whole-plant and separated plant components

(2) A 48 h incubation using DaisyII incubator jars to determine NDFD of whole-plant and separated plant components

(3) A 120 h incubation using a fully automated system (Gas Endeavour) to determine the kinetic of fibre fermentation of soybean and maize silages

(4) A 288 h incubation using DaisyII incubator jars to determine uNDF of faeces and TMRs

NEl was estimated according to the gas production (GP) method of Menke and Steingass (1988), working with three replicates per sample, correcting for standards and blank (i.e. syringe without sample). Rumen fluid was collected from three fistulated dry Italian Friesian cows fed a diet composed of meadow hay, maize silage, ryegrass hay, SBM, maize meal, and mineral and vitamin integration (676, 96, 77, 70, 54 and 25 g/kg DM, respectively). The donor animals were handled as outlined by the Directive 2010/63/EU on animal welfare for experimental animals, according to the University of Milan Welfare Organism (OPBA) and with authorization number 904/2016-PR from the Italian Ministry of Health. The cows were fed the TMR twice daily (0700 and 1900 hours) to achieve a DMI of 8 kg/d. Rumen liquor was collected two h after the morning feeding. The incubation was run in 100-ml glass syringes (Haberle Labortechnik, Germany), according to Menke and Steingass (1988). Equation 12b (Menke and Steingass 1988) was used to estimate NE_l for seeds, while equation 12c was used for the other components and the whole-plant.

In the second incubation, NDFD was evaluated at 48 h *in vitro* incubation using the DaisyII incubator jars (Ankom Technology, Macedon, NY, USA). For each sample, 0.500 g was weighted, with three replicates per sample, in F57 bags. The bags were pre-treated, washing them with NDS and α-amylase before being incubated, according to Battelli et al. (2020). Each jars of DaisyII incubator contained standards and blanks (i.e. bags without sample). Rumen fluid and the fistulated cows were treated as explained above. The buffer was composed by two solutions as reported by Ankom protocol. The inoculum was mixed with the buffer in a ratio of 450 g/L, for a total of 1.6 L, while rumen fluid was added at a dose of 400 mL/jar, using a 1:4 ratio with the buffer into each pre-warmed (39°C) jar. After 48 h of incubation, jars were emptied and the F57 bags were rinsed thoroughly with cold tap water and analysed for aNDFom content using the Ankom200 fibre analyser.

The kinetic of fibre fermentation of soybean silage and maize silage samples was assessed using a fully automated system (Gas Endeavour, Bioprocess Control AB, Lund, Sweden) for the real-time monitoring of GP in rumen fermentation batch processes. The substrates analysed consisted of 2 (±0.01) g of pure NDF residue, previously obtained treating the samples with neutral detergent solution using the Ankom200 fibre analyser and filter bags (Sefar Petex® 12x6 cm; 15μm pore size) with 3.75 g of sample. Three replicates per sample for each period were used. The incubation was run into 500 ml reactors and blanks in triplicate were also included. Particularly, the final incubation medium contained the buffer solution, prepared according to Menke and Steingass (1988), and rumen liquor in a 2:1 ratio, treating the rumen liquor as explained above. Each batch contained 300 ml of the medium, and was kept in continuous stirring at 39°C. The incubation lasted 120 h, with continuous and automated measurement and registration of the gas produced, normalized at 0°C and 101.3 kPa. Potential GP (pGP) (mL/g NDF) at time t was estimated following the model derived from that reported by McDonald (1981), as explained in the equation below:

$$\mathrm{pGP} = \mathrm{b} \times \left(1 - \mathrm{e}^{-\mathrm{k_{GP}} \times (\mathrm{t}-\mathrm{l})}\right)$$

with b: potential GP (mL/g NDF); k_{GP}: GP rate (%/h); t: incubation time (h); l: lag phase (h).

The model for pGP kinetic was fitted to net gas volume data using the algorithm of Levenberg Marquardt employed in the NLIN procedure of SAS 9.4.

To assess digestibility of DM, OM, CP, and NDF (DMD, OMD, CPD, and NDFD, respectively), the undigested NDF (uNDF) of TMRs and faeces, estimated at 288 h in vitro incubation, was used as internal marker according to the following equations:

$$\mathrm{DMD} = 100 - \left(100 \times \frac{\%\mathrm{uNDF\ in\ TMR}}{\%\mathrm{uNDF\ in\ faeces}}\right)$$

$$\mathrm{NutrientD\ (OMD, CPD, and\ NDFD)} = 100 - \left(100 \times \frac{\%\mathrm{uNDF\ in\ TMR}}{\%\mathrm{uNDF\ in\ faeces}} \times \frac{\%\mathrm{Nutrient\ in\ faeces}}{\%\mathrm{Nutrient\ in\ TMR}}\right)$$

Faecal samples were pooled per period for each cow, and SBS and CON TMRs and the silages where pooled per period. In addition, sample of soybean silage and maize silage were incubated to determine their uNDF and potentially degradable NDF (pdNDF) at 288 h, according to the following equation:

$$\mathrm{pdNDF} = 100 - \left(100 \times \frac{\%\ \mathrm{uNDF}}{\%\ \mathrm{NDF}}\right)$$

Incubations were conducted as explained above, using the DaisyII incubator jars.

Statistical analysis

Using the proc univariate procedure (normal option) of SAS 9.4, the Shapiro-Wilk normality test was used to determine whether or not the residuals were normally distributed. All of them resulted normally distributed ($P > 0.05$). The data collected were statistically analysed by the proc mixed procedure of SAS 9.4, with the following model:

$Y_{ijklm} = \mu + SEQ_i + P_j + T_k + LACT_l + COW_m(SEQ_i) + \varepsilon_{ijklm}$

where Y_{ijklm} is the dependent variable; μ is the overall mean; SEQ_i is the treatment sequence effect (i=1, 2); P_j is the period effect (j = 1, 2); T_k is the treatment effect (k = 1, 2); $LACT_l$ is the number of lactation effect (l = 1, 7); COW_m is the random animal effect (l = 1, 36), and ε_{ijklm} is the residual error.

The data regarding the chemical analysis and the nutritive value of whole-plant and separated plant components were statistically analysed by the proc glm procedure of SAS 9.4, with the following model:

$Y_{ij} = \mu + C_i + F_j + \varepsilon_{ij}$

where Y_{ij} is the dependent variable; μ is the overall mean; C_i is the plant components effect (i=1, 5); F_j is the field plot effect (j = 1, 5), and ε_{ij} is the residual error.

Least squares means estimates are reported. For all statistical analyses, significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

Results

Nutritive value of whole-plant soybean and plant components

The chemical composition of the soybean and maize silages used in the experiment is shown in Table 2. Compared to maize silage, the forage with the highest inclusion in CON diet, soybean silage had higher pH and lower concentration of lactic and acetic acid while butyric acid was low for both silages. Protein fractions A and B2 were higher for soybean silage than for maize silage.

Relative contribution to total DM, chemical analysis and nutritive value of whole-plant soybean and plant components at harvesting are reported in Table 3. More than 1/3 of soybean whole-plant DM was represented by the stalk and another 1/3 by the seeds. These two components were characterized by the lowest and the highest nutritive value. Stalks had the highest content (% DM) of NDF with the lowest NDFD (% of NDF) while seeds the lowest NDF concentration and the highest digestibility of NDF ($P < 0.001$). Seeds had the highest concentration (% DM) of CP followed by leaves; EE concentration (% DM) was highest ($P<0.001$) for seeds as well, while it was much lower for the other components (in particular, below 1% for pods and stalks). Table 4 reports the data concerning ruminal fermentation of NDF of soybean and maize silages. Since the replicates were not independent, no statistical analysis was applied. The NDF of soybean silage was fermented faster than that of maize silage (k_{GP} of soybean silage was higher than maize silage) and the lag phase was shorter. However, the NDF of soybean silage was less fermentable because the potential GP (mL/g of NDF) was lower and this result was found also after 288 h of incubation (pdNDF).

Dry matter intake and milk production

The partial substitution of SBM with soybean silage did not affect milk production or FPCM (Table 5). Milk production was, on average, 33.0 and 34.5 kg/d, respectively, for milk and FPCM, and both of them were not significantly different between the dietary treatments ($P = 0.377$ and $P = 0.474$, respectively). Also dairy efficiency and DMI, either estimated through the model of Dórea et al. (2017) or with the NRC (2001) equation, were not affected by the treatment. Considering DMI estimated with the model of Dórea et al. (2017), the average values of dairy efficiency and DMI of the two treatments were 1.40 and 23.7 kg ($P = 0.783$ and $P = 0.659$, respectively). The fat yield was higher for cows fed SBS than CON ($P = 0.024$), but the treatment did not affect milk fat concentration ($P = 0.806$). The SBS diet resulted in lower milk protein concentration ($P < 0.001$) and higher milk urea than CON ($P = 0.002$). As for protein, milk casein concentration was higher for CON ($P < 0.001$) but, when expressed as a percentage of total N, was higher for SBS ($P = 0.004$).

Digestibility

The values of total tract digestibility of cows fed the two dietary treatments are reported in Table 6. The SBS resulted in lower digestibility ($P < 0.001$) than CON for DM, OM, and NDF. The cows fed CON diet had a tendential (P = 0.065) higher CP digestibility than SBS diet.

N balance

There was a tendency (P = 0.098) for higher N intake for cows fed SBS diet than CON (Table 7). The faecal N excretion was higher for SBS, but the difference was significant only when expressed in g/d (P = 0.013). The diet affected urinary N excretion when expressed both in g/d and as % of N intake (P < 0.001 and P = 0.005, respectively), with higher values for SBS than CON. Milk N excretion (g/d) was not different between treatments; however, N efficiency (N milk/N intake × 100) was higher for CON than SBS (P = 0.003). The soybean silage diet resulted in a higher N mobilization than CON (P = 0.006 and P = 0.011, respectively, for g/d and percentage balance). On the opposite, cows fed CON stored N (Table 7).

Creatinine and PD

Urine volume, urine content of N (%), and creatinine, uric acid, and allantoin (mmol/L) were not affected by the treatment. The same result was found when the excretion of creatinine, uric acid, and allantoin was expressed in mmol/d (Table 8).

Discussion

Nutritive value of whole-plant soybean and plant components

The soybean silage used in the present study proved to be a good source of CP and EE. These two chemical parameters are higher than those of the soybean silage used in the experiments of Silva et al. (2021), Ghizzi et al. (2020), and Vargas-Bello-Pérez et al. (2008), where the focus was to evaluate its potential in substitution of another forage and not as a replacement of protein source. In the soybean silage used in the present study, NDIP and ADIP concentrations were lower in comparison with the CNCPS feed bank, (1.84% and 1.76% vs. 4.33% and 2.14% of DM in the CNCPS), while NH_3 was higher (11.2% vs. 8.6% of total N). In Silva et al. (2021), NDIP (2.63%) was lower than the CNCPS one as well. As expected, most of the protein in the soybean plant derived from the seeds; however, the leaves, with 20.8% of CP on DM, also contributed significantly to the total plant CP. Overall, the chemical composition of soybean components in the present study and that of R6 stage soybean silage in Spanghero et al. (2015) are very close to each other, except DM concentration, because in the present experiment soybean was ensiled without a preliminary wilting phase. However, NDFD resulted in being closer to the R5 stage soybean of Spanghero et al. (2015), especially for stalks (19.3% R5 and 8.2% R6 vs. 21.3% in the present study) and whole-plant (38.8% R5 and 46.5% R6 vs. 38.6% in the present study). Whole-plant NDFD was in line with what was found by Mustafa and Seguin (2003) for soybean harvested between R5 and R6 stage (35.5%), measured in situ up to 96 h incubation. Regarding gas production, the present data for whole-plant resulted higher than the study mentioned above for both 6 h and 24 h incubations.

Dry matter intake and milk yield

The lactation trial results demonstrated that soybean silage used as a self-produced protein source at the inclusion level of 12.4% of diet DM did not hamper milk production. Differently, the results reported by Ghizzi et al. (2020) (where soybean silage was included as 0, 8, 16, and 24% of diet total DM) and Vargas-Bello-Pérez et al. (2008) (inclusion was 36% of diet total DM), showed a reduction of milk yield with a reduction of DMI, due to a greater NDF concentration in the diet (Vargas-Bello-Pérez et al. 2008) or to greater intakes of longer feed particles and EE for soybean silage diets (Ghizzi et al. 2020). Intake was reduced in the study of Silva et al. (2021) (with soybean silage representing the 8% of diet DM), because of higher proportion of long particles (> 19 mm) in the soybean silage diet; however, milk production was not affected. In the present study, the NDF values were 30.4% for SBS and 30.6% for CON while in Vargas-Bello-Pérez et al. (2008), NDF concentrations were 36.7% for the soybean silage diet and 34.1% in the lucerne control diet. The EE content of the diets of the present study was similar (3.33% of SBS and 2.72% of CON), while in Ghizzi et al. (2020), the EE of the experimental diets ranged between 3.33% and 4.45%. However, the higher EE concentration of soybean silage of the present study (7.28% on DM) may be responsible for the higher yield of milk fat of SBS treatment.

In the present study, it was not possible to determine the individual DMI gravimetrically. However, according to the conclusions of the meta-analysis of Dorea et al. (2017), PD can be used as an alternative method to estimate feed intake in dairy cattle in research trials, so individual DMI was estimated through it. This method takes into account factors that directly influence feed intake (i.e., fat corrected milk, body weight and the week of lactation). Moreover it considers the fact that the DM ingested in turn influences the microbial development and consequently the microbial protein yield with the associated urine PD (Dórea et al. 2017). The equation n. 11 (Dórea et al. 2017) was selected because of the lowest root mean squared error (0.49) and one of the highest R^2 (0.91) among the models evaluated for dairy cattle. However, being an estimation, the values obtained have to be considered with caution. The prediction of microbial protein yield might be improved also considering diet composition, rather than just DMI (Oldick et al. 1999). Secondly, the urinary recovery of duodenal purines, like allantoin, might affect the performance of the model used (Gonzalez-Ronquillo et al. 2004).

Nevertheless, this method gave reasonable results if compared with DMI obtained through the NRC equation: for the two diets, the estimation through PD gave, on average, an intake 5.6% lower than that predicted by the NRC equation. In both estimations, DMI was not affected by the diet. By contrast, in the study of Tabacco et al. (2019), the diet with the inclusion of soybean silage (8.7% on total DM) resulted in higher DM intake in comparison with control (23.2 vs. 22.3 kg/d). Creatinine, uric acid, and allantoin were not statistically different according to the dietary treatment. The daily production amounts of these metabolites were within the ranges found by Dórea et al. (2017), namely 96-208 mmol/d for creatinine, 5-118 mmol/d for uric acid, and 169-713 mmol/d for allantoin.

Milk quality

The main effect on milk quality is related to milk N compounds. Lower milk protein percentage and higher milk urea of the SBS diet can be associated with an unbalanced ratio of protein/energy provided with the diet (Oltner and Wiktorsson 1983). In particular, the rumen degradability of protein is high for legume silages, so, in order to incorporate more efficiently this dietary N into microbial protein, it is advisable to increase the concentration of readily fermentable carbohydrates, as found by Broderick (2003). High degradable N is also due to protein degradation during silage storing (Dewhurst et al. 2003). These results are in agreement with the findings of Ghizzi et al. (2020), where milk protein concentration decreased linearly with increasing inclusion levels of soybean silage, probably because of lower CPD, (from 75.7% with 0% soybean silage inclusion to 67.6 % with 24% inclusion). However, the authors found that milk urea nitrogen (MUN) was numerically but not significantly higher. In Silva et al. (2021), no statistical differences were found for milk protein, milk protein yield, and MUN between the soybean silage and the maize silage diet. In contrast, Tabacco et al. (2019) found higher milk protein for soybean silage (3.60% vs. 3.45%) and no difference in milk urea. However, the two diets of the above cited work had lower protein concentration (13.9% for control and 13.3% for the soybean silage diet). Moreover, the two experimental diets had high but very similar inclusions of maize silage and high moisture maize (on average for the two diets: 29.6% and 12.3% of total diet DM respectively). Higher N excretion through urine and higher, although not alarming, concentrations of acetone and BHB in the milk of the cows fed SBS also confirmed the insufficient energy provided by SBS. Vargas-Bello-Pérez et al. (2008) found higher MUN content with soybean silage too. However, higher MUN has to be taken into account when SBM is reduced into the diet in favour of other legume protein source (Volpelli et al. 2009a; 2009b). Nevertheless, protein yield was not affected by the treatment in the present study.

Digestibility

The cows fed the SBS diet had a lower total tract digestibility than CON. This result is in agreement with Ghizzi et al. (2020), who found decreasing values of DMD, OMD, and NDFD with increasing inclusion of soybean silage. By contrast, in Silva et al. (2021), the diet with the inclusion of soybean silage was not less digestible than the one based solely on maize silage as forage source; even NDFD was only numerically different despite low NDFD for soybean silage (27.4%). Moreover, in Ghizzi et al. (2020), soybean was harvested at stage R5.5 (with silage DM of 23.6%) while in the present study at R6 (24.5% DM), which should guarantee higher NDF and CP digestibility (Spanghero et al. 2015). However, the value of NDFD (31.5%) in the present study is very close to the value found by Vargas-Bello-Pérez et al. (2008) (31.2% for NDF ruminal digestibility) in a diet where soybean silage was included for 36% of total DM. As hypothesized by Ghizzi et al. (2020), poor NDFD was probably the main driver in reducing the overall DMD of SBS compared to CON. Tabacco et al. (2019) found lower in vitro NDFD with advancing phenological stage (from 53.6% of R4-5 to 51.5% of R7-8) but associated with higher NE_l (from 6.11 to 6.51 MJ/kg DM); by contrast, Spanghero et al. (2015) reported an increase of NDFD with advancing phenological stage (from 31.9% of R4 to 46.5% of R6). A possible explanation can be given considering the different plant components, with lower NDFD found for stalks. Another option could be using lower size varieties. In Tabacco et al. (2013), the authors found higher NDFD for silages of a variety of soybean with low size plant compared to one with medium-tall size plant (51.6% vs. 46.4%, on average). This could be due to the lower contribution of stalks on total DM in favour of pods, as suggested by higher CP content of the low size plant (22.8% vs. 19.5%), and by the more lignified fibre of medium-tall size plant (lignin concentration was 8.7% vs. 6.4% of the low size variety). Low NDFD was confirmed also considering NDF fermentation kinetic. The values k_{GP} in the present study was lower than the k_d found by Silva et al. (2021) (4.74%/h vs. 6.74%/h), but in line with Vargas-Bello-Pérez et al. (2008) and Mustafa and Seguin (2003) (4.8%/h and 5.1%/h, respectively). In Mustafa and Seguin (2003), the lag phase was (0.1 h) shorter than the one found here (1.41 h). Regarding OMD, Silva et al. (2021) found a value for the soybean silage diet (69%) not far from the one reported here. Another key factor in improving the quality of whole-plant soybean silage is CPD, especially if the goal is to increase the farm protein self-sufficiency. In the work of Spanghero et al. (2015), CPD was improved by advanced maturity stage at harvest time (i.e., at R6), due to the higher protein accumulation in the pods, as supposed by the authors. It can be assumed that delaying the harvest of soybean in the present study would have improved CPD as well, because the present values of NDFD are closer to R5 than to R6 soybean of the work of Spanghero et al. (2015). In the work of Rigueira et al. (2015), digestibility of DM, NDF, CP, and NFC of a diet containing soybean silage for beef cattle was improved by treating chopped soybean with microbial inoculant and molasses before ensiling. The authors explained this result by a better fermentation of the treated silage, which led to lower losses of cellular content, more digestible than the cell wall components (fibre). In the present study, whole-plant soybean silage pH after 50 d storage was 5.30, very close to Vargas-Bello-Pérez et al. (2008) (5.29), but higher compared to the pH of soybean silage in the study of Rigueira et al. (2015) or in the study of Touno et al. (2014) (4.78). Even if this pH value may suggest poor fermentation of soybean silage in the present study, no sign of spoilage was detected and the NDF content was in line with Touno et al. (2014) (43.2% vs. 45.3% on DM) and lower than Rigueira et al. (2015) (52.4% on average).

Nitrogen balance

Cows fed SBS had a lower dietary N use efficiency compared to CON. This could be due to unbalanced ratio protein/energy and a numerically lower CPD. Cows fed SBS had higher N faecal excretion (in g/d) and N urine excretion (as % of N intake). Higher excretion of N is detrimental for the environment as well; however, it has to be taken into account a reduction of N coming from outside the farm gate due to a lower use of SBM. According to the estimation made by Wilkinson and Young (2020), 700,000 tonnes of N coming from imported SBM have been excreted by livestock in EU in 2018/2019. The negative, even if close to zero, N retained value for SBS caused the mobilization of body reserve, without affecting milk production. Maybe this was because the cows were far from the lactation peak (159 DIM, on average at the beginning of the study), so with lower metabolizable protein requirements. Long-term experiments could better elucidate if the negative N balance found with the present experimental conditions could negatively impact milk production.

Conclusions

Whole-plant soybean silage proved to be an adequate forage and protein source to be included in the ration of lactating cow at 12.4% of the DM, allowing a reduction of one-third of SBM (more than 1 kg/head per day), without affecting feed intake and milk production. Thus, environmental sustainability of milk production can be enhanced thanks to protein source grown on farm. Future research should be aimed at quantifying the environmental impact of SBS compared to CON. Possible limitations of soybean silage are that digestibility and protein use efficiency have to be improved in order to fully exploit its potential. More studies are advisable to better understand the effect of management and harvest practices aimed at increasing digestibility and to improve the protein/energy ratio in the diet, for example including higher amount of water soluble carbohydrates sources like sugarcane molasses.

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Disclosure statement

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author, A. R. G., upon reasonable request.

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Table 1. Composition of a diet with partial substitution of soybean meal with soybean silage (SBS) and the control diet with soybean meal (CON) (% of DM).

Item	**SBS**	**CON**
High moisture maize	15.6	0.00
Soybean silage	12.4	0.00
Barley silage	10.2	10.3
Lucerne hay	9.43	9.52
Maize silage	9.22	25.5
Maize meal	8.72	16.4
Flaked maize grain	7.27	7.31
Soybean meal	6.91	10.7
Sunflower meal	4.94	4.97
Molasses cane	4.81	4.83
Barley grain	2.79	2.80
Wheat straw	1.95	1.95
Maize gluten feed	1.51	1.52
Wheat shorts	1.20	1.21
Wheat middlings	1.05	1.06
Sodium bicarbonate	0.76	0.76
Soybean oil	0.62	0.62
Calcium carbonate	0.31	0.31
White salt	0.11	0.11
Minvit[1]	0.09	0.09
Magnesium oxide	0.05	0.05

[1] Each kg contained: 31 g Fe, 70.5 g Zn, 30.4 g Mn, 100 mg Se, 2 g I, 60 mg Mo, 6.9 g Cu, 500 mg beta carotene, 4,000,000 IU Vitamin A, 800,000 IU Vitamin D3, 20,500 IU Vitamin E, 2450 IU Vitamin B1, 343 IU Vitamin B6, 20 IU Vitamin B12, and 52,000 IU Vitamin PP.

Table 2. Chemical analysis of a diet with partial substitution of soybean meal with soybean silage (SBS), the control diet with soybean meal (CON), soybean silage and maize silage used in these diets.

		Soybean silage	**Maize silage**	**SBS**	**CON**
DM	% AF	24.5	33.1	48.9	49.6
Ash	% of DM	9.00	4.33	6.38	5.90
OM	% of DM	91.0	95.7	93.6	94.1
CP	% of DM	23.0	8.03	15.2	14.7
Sol CP	% of CP	61.1	63.1	29.8	21.6
EE	% of DM	7.28	3.17	3.33	2.72
NDF	% of DM	43.2	44.7	30.4	30.6
ADF	% of DM	33.3	25.5	18.9	18.8
ADL	% of DM	7.09	3.63	3.89	3.81
NDIP	% of DM	1.84	1.35	1.34	1.32
ADIP	% of DM	1.76	1.14	0.96	1.03
NFC	% of DM	17.5	39.8	44.8	46.0
Lactic acid	%	5.27	8.16		
Acetic acid	%	0.88	1.92		
Butyric acid	%	0.28	0.33		
pH		5.30	3.81		
N-NH_3	% of total N	11.2	7.10	13.9	15.1

Table 3. Chemical analysis and nutritive value of whole-plant soybean and plant components.

		Pods	**Leaves**	**Seeds**	**Stalks**	**Whole-plant**	**s.e.**	***P-value***
% of whole-plant DM		15.2^{b}	15.7^{b}	34.1^{a}	35.0^{a}		1.80	<0.001
DM	% AF	24.7^{c}	30.5^{b}	41.9^{a}	23.4^{c}	29.5^{b}	0.60	<0.001
Ash	% DM	8.67^{b}	11.2^{a}	5.38^{d}	8.81^{b}	7.87^{c}	0.18	<0.001
CP	% DM	12.0^{c}	20.8^{b}	37.6^{a}	9.09^{d}	20.7^{b}	0.54	<0.001
EE	% DM	0.96^{d}	2.35^{c}	12.2^{a}	0.64^{d}	5.76^{b}	0.41	<0.001
aNDFom	% DM	51.2^{b}	30.3^{d}	19.1^{e}	64.1^{a}	38.4^{c}	0.90	<0.001
ADFom	% DM	39.0^{b}	16.2^{d}	12.8^{e}	52.2^{a}	29.3^{c}	0.78	<0.001
ADL	% DM	6.63^{b}	4.28^{c}	0.25^{d}	10.5^{a}	4.72^{c}	0.34	<0.001
NDFD	%NDF	38.0^{c}	55.0^{b}	92.5^{a}	21.3^{d}	38.6^{c}	2.05	<0.001
NE$_l$[1]	MJ/kg DM	4.70^{b}	4.95^{b}	7.22^{a}	3.21^{c}	5.13^{b}	0.2	<0.001
Gas[2] 6 h	ml	22.2^{a}	22.4^{a}	18.1^{b}	14.5^{c}	19.4^{ab}	1.16	<0.001
Gas[2] 24 h	ml	42.3^{a}	38.2^{ab}	40.7^{a}	26.3^{c}	34.7^{b}	2.00	<0.001
Gas[2] 48 h	ml	47.7^{a}	43.7^{ab}	44.9^{ab}	31.0^{c}	39.4^{b}	2.57	<0.001

^{abcde}Means in the same row with different superscripts are statistically different at $P<0.05$

[1] NE$_l$ = -1.04 + 0.1195 × Gas production + 0.0051 × CP + 0.0152 × EE, for seeds; NE$_l$ = 0.81 + 0.0816 Gas production + 0.0046 × CP + 0.0135 × EE, for pods, leaves, stalks, and whole-plant.

[2] Gas production from 200 mg DM

Table 4. Ruminal fermentation of NDF of soybean silage and maize silage.

		Soybean silage		**Maize silage**	
		mean	**s. d.**	**mean**	**s. d.**
Gas Endeavour					
b[1]	mL/g NDF	98.5	15.1	162	5.47
k_{GP}[2]	%/h	4.74	0.36	3.01	0.18
l[3]	h	1.41	0.72	3.50	0.98
DaisyII					
pdNDF 288 h	% NDF	54.1	0.49	83.5	0.81

[1] b: potential gas production.

[2] k_{GP}: gas production rate.

[3] l: lag phase.

Table 5. Intake, milk yield and composition of cows fed a diet with partial substitution of soybean meal with soybean silage (SBS) and the control diet with soybean meal (CON).

		SBS	**CON**	**s.e.**	***P-value***
DMI	kg/d	23.8	23.6	0.511	0.659
DMI (NRC)[a]	kg/d	25.3	24.9	0.509	0.263
Milk	kg/d	33.2	32.7	1.68	0.377
FPCM[b]	kg/d	34.7	34.2	1.48	0.474
Dairy efficiency		1.40	1.39	0.053	0.783
Fat	%	4.46	4.44	0.146	0.806
Fat yield	kg/d	1.50	1.41	0.069	0.024
Protein	%	3.43	3.55	0.060	<0.001
Protein yield	kg/d	1.13	1.15	0.050	0.378
Lactose	%	5.00	4.98	0.037	0.261
Linear score		1.76	1.72	0.445	0.825
Urea	mg/dL	30.5	28.7	0.743	0.002
Casein	%	2.70	2.78	0.050	<0.001
Casein	% of total N	78.7	78.4	0.221	0.004
Acetone	mM	0.016	0.006	0.004	0.008
BHB	mM	0.034	0.019	0.005	<0.001
Live weight	kg	645	642	17.4	0.444

[a] FPCM = Milk × (0.122 × fat + 0.072 × protein + 0.052 × lactose) (adapted from NRC 2001).

[b] DMI (NRC) = (0.372 × (fat corrected milk) + 0.0968 × (body weight) ^ 0.75) × (1 - EXP(-0.192 × ((week of lactation) + 3.67))) (adapted from NRC 2001).

Table 6. Total tract digestibility of a diet with partial substitution of soybean meal with soybean silage (SBS) and the control diet with soybean meal (CON).

		SBS	**CON**	**s.e.**	***P-value***
DMD	% of DMI	65.2	68.6	0.491	<0.001
OMD	% of OM intake	66.4	69.8	0.460	<0.001
NDFD	% of NDF intake	31.5	38.8	0.776	<0.001
CPD	% of CP intake	60.0	62.5	1.05	0.065

Table 7. N balance of cows fed a diet with partial substitution of soybean meal with soybean silage (SBS), and the control diet with soybean meal (CON).

		SBS	**CON**	**s.e.**	***P-value***
N intake	g/d	572	555	12.2	0.098
N faeces	g/d	229	207	9.12	0.013
	% of N intake	40.0	37.5	1.53	0.065
N urines	g/d	183	158	8.24	0.001
	% of N intake	32.3	28.9	1.42	0.005
N milk	g/d	178	181	7.89	0.378
	% of N intake	31.3	32.7	1.04	0.003
N retained	g/d	-17.5	9.3	12.1	0.006
	% of N intake	-3.53	0.92	2.19	0.012

Table 8. Urine volume, protein and creatinine, uric acid and allantoin excretion from cows fed a diet with partial substitution of soybean meal with soybean silage (SBS) and the control diet with soybean meal (CON).

		SBS	**CON**	**s.e.**	***P-value***
Urine	L/d	19.3	19.1	1.70	0.912
N	%	0.985	0.923	0.053	0.179
Creatinine	mmol/L	6.04	6.57	0.445	0.111
	mmol/d	110	110	2.98	0.444
Uric acid	mmol/L	1.27	1.41	0.121	0.180
	mmol/d	23.8	23.7	2.05	0.965
Allantoin	mmol/L	17.4	19.5	1.11	0.068
	mmol/d	325	334	19.0	0.589

7. Experiment 5

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Partial replacement of soybean meal with soybean silage and responsible soybean meal in lactating cows diet: part 2, environmental impact of milk production

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Partial replacement of soybean meal with soybean silage and responsible soybean meal in lactating cows diet: part 2, environmental impact of milk production

Soybean meal, the main protein source for livestock in Italy, is associated with high environmental impact in terms of land use change. Thus alternative protein sources are advisable.The study aimed to evaluate through a Life Cycle Assessment (LCA) approach the environmental impact of milk production systems characterized by different diets of lactating cows including different sources of soybean. Four scenarios were identified: 1) conventional soybean meal (CON), 2) conventional soybean meal and soybean silage (SBS), 3) responsible soybean meal defined by the FEFAC guidelines (CON + RSM), 4) soybean silage and responsible soybean meal (SBS + RSM). Inventory data were derived from a previous in vivo trial on lactating cows and farmer interviews. Secondary data were obtained from the ECOINVENT® and the Agri-footprint databases. The LCA was performed using the SimaPro V 8.3.

Soybean silage showed higher global warming potential (GWP), marine eutrophication and human toxicity compared with lucerne hay, the most utilized self-produced protein feed, due to the high contribution of mechanical operations in the field. The GWP of milk (kg CO_2eq/kg FPCM) decreased from 1.38 of the CON scenario to 1.17 of SBS and 1.13 of CON + RSM; the best result was obtained by combining soybean silage with responsible soybean meal: 1.01. Furthermore, the scenarios using RSM reduced agricultural land occupation and natural land transformation. The inclusion of SBS and RSM is an interesting option to reduce environmental impact of milk production, maximizing yields of DM and CP per hectare and representing an alternative protein source.

Keywords: soybean silage, responsible soybean meal, LCA, milk

Highlights

The ration of dairy cows represents one of the main causes of the environmental impact of the livestock sector due to the impact for feed production (forage and concentrate)

Feeding soybean meal as protein source has high environmental impact since it is linked with deforestation in South America

Alternative protein sources like soybean silage and soybean meal produced sustainably could reduce the environmental impact of the sector

Introduction

According to the international bibliography (e.g., Laca et al. 2020; Lovarelli et al. 2019), the main cause of greenhouse gas (GHG) emissions at the dairy farm level is enteric fermentation, followed by feed production and manure management. Feed emission is, precisely, the second largest category of emissions, contributing about 36% to milk emissions (Gerber et al. 2013). Regarding feed production, the trend in Northern Italy is to satisfy the energy requirement of the herd through the self-production of whole-plant maize silage (Gislon et al. 2020a) while purchasing protein sources from the market (Borreani et al. 2013), in particular, soybean meal. The use of soybean meal is positively correlated to the environmental impact of the ration (Gislon et al. 2020a), mainly for its geographical origin. According to ASSALZOO (2020) data, 50% of soybean meal used in Italy is imported, and 33.8% is produced locally from imported seeds, mainly from Argentina, the USA, and Brazil. The other 16.2% is produced from Italian soybean (Eurostat 2020).

Soybean is grown in South America on former virgin lands. In Argentina and Brazil it was estimated that 9% and 15.6% of the new soybean area was associated with deforestation, respectively (Malins 2020), clearing forests and savannahs in Argentina, and the Amazon forest and Cerrado in Brazil, with loss of biodiversity and C stock in the soil (Bickel and Dros 2003). Decreasing the use of soybean meals, thus, may be considered as an effective strategy to enhance the sustainability of the dairy cow livestock system (Gislon et al. 2020a) and, in this regard, the use of self-produced high protein forages could reduce the reliance of farms on imported soybean meal (Tabacco et al. 2018). Furthermore, on-farm legumes cultivation, in rotation with grain crops, has other environmental benefits, in particular, the potential to reduce N fertilization due to N fixation capacity of these species (Nemecek et al. 2008), to break the life cycle of crop-specific pathogens, pest, and weeds compared to monoculture and to increase soil organic carbon (Kirkegaard et al. 2008; Stagnari et al. 2017). In the study of Zucali et al. (2018), the scenario with protein-rich forage in the cropping system (i.e., lucerne preserved as hay and soybean preserved as silage) was judged the best one for the lowest environmental impact per unit of product (fat and protein corrected milk, FPCM), in terms of acidification, eutrophication, and non-renewable fossil energy use, besides showing the highest feed self-sufficiency and reduction of chemical N fertilization. The inclusion of soybean silage in lactating cows total mixed ration (TMR) was investigated in the companion paper (Rota Graziosi et al. submitted), and the authors found promising results, as no differences in DMI and milk production were found between a control diet (based on soybean meal) and a diet with the inclusion of soybean silage in partial substitution of soybean meal. On the opposite, in Comino et al. (2018), cows fed a diet with soybean silage in complete substitution of soybean meal and cotton seed had lower milk yield, but higher milk fat and protein concentrations.

Another opportunity, emerging over the last years, to enhance the sustainability of the dairy cows sector is the possibility of using a ‘responsible soy’ for livestock feeding. The European Feed Manufacturers' Federation (FEFAC) (FEFAC and ITC 2021), indeed, suggests using responsible soy, defined as soy imported in Europe from production sites that follow sustainability guidelines. To be considered responsible, a soybean crop has to satisfy several sustainability criteria: legal compliance, working conditions, respect and protection of the environment, implementation of good agricultural practices, legal use of lands, and the protection of community relations, respecting the reserve or conservation areas. Thus, in terms of environmental impact, the main difference between responsible and conventional soy is that the production of the second one is not associated with Land Use Change (LUC). The importance and urgency of these measures are stressed, considering that LUC accounts for more than 50% of the carbon footprint of soybean imported in Europe from Brazil (Escobar et al. 2020). The EU and other international buyers of Brazilian commodities are aware of this environmental threat. Indeed, 38% of the soybean meal consumed in the EU in 2018 was in compliance with FEFAC soy sourcing guidelines, and 19% of it was defined as ‘deforestation-free’ (Eurostat, 2020). The certified volume of responsible soy increased 4.5 times from 2013 to 2020 (4.7 million tons in 2020) (https://responsiblesoy.org/).

Our hypothesis was that the substitution of conventional soybean meal with alternative protein sources, like soybean silage and responsible soybean meal, could reduce the environmental impact of dairy cows diet. To the best of our knowledge, a combination of these two protein sources was not investigated before. Thus, the aim of this study was to evaluate, through a Life Cycle Assessment (LCA) approach and also based on in vivo results reported in companion paper (Rota Graziosi et al. submitted), the environmental impact of four lactating cow rations. These diets were characterized by different sources of soybean: conventional soybean meal, soybean silage and responsible soybean meal.

Material and methods

The experimental diets

The yield at field of soybean silage was compared with that of other forage sources included in lactating cow diet (Table 1), both in terms of dry matter (DM) and crude protein (CP). The forages were included into two lactating cows TMR (CON and SBS, Table 2) characterized by different sources of soybean, i.e. conventional soybean meal and soybean silage. In addition, two different TMR were fed replacing soybean meal with responsible soybean meal. So, four scenarios were identified: 1) conventional soybean meal (CON, control diet), 2) conventional soybean meal and soybean silage (SBS, soybean silage), 3) responsible soybean meal as defined by the FEFAC guidelines (CON + RSM, control diet plus responsible soybean meal), or 4) soybean silage and responsible soybean meal (SBS + RSM, soybean silage plus responsible soybean meal). Soybean oil was also either from conventional soybean (for CON and SBS diets) or from responsible soybean (for CON + RSM and SBS + RSM diets). The environmental impact of milk production systems in the four scenarios were investigated based on the results reported in companion paper (Rota Graziosi et al. submitted). In this latter study, a total of 36 Holstein cows were involved in the study, and data regarding diet composition, intake, milk production and quality, digestibility, and N balance where collected and used in the present paper.

Life Cycle Assessment

The environmental sustainability was performed through LCA method, structured following ISO 14040-compliant and ISO 14044-compliant LCA methodology (ISO 2006a, b).

Goal and scope definition

The goals of this LCA study were to quantify the environmental sustainability of different forage sources, individual daily administered TMR, and milk production to evaluate the possible effects of the use of different soybean sources in lactating cow diets.

Functional units, allocation and system boundaries

In order to compare the sustainability of soybean silage with that of other forage sources, 1 ton of DM and 1 ton of CP were considered as functional units (FU). In addition, the individual daily supplied diet was considered as FU. For the analysis of different milk production scenarios, the considered FU was 1 kg of fat and protein corrected milk (FPCM; 4.0% fat and 3.3% protein), calculated as suggested by the International Dairy Federation (IDF 2015). Therefore, the allocation between FPCM and meat was calculated using a physical method (IDF 2015).

An attributional approach, which considered from cradle to farm gate system boundaries, was adopted. All the inputs (e.g., off-farm feeds and bedding, machinery, fuel, lubricants, electricity, organic and mineral fertilizers, pesticides, plastics, and water), and outputs (i.e., emissions to the air, soil and water, milk, and meat) involved in the production process were considered within the system boundaries (Figure 1).

Life cycle inventory (LCI)

Primary data of the foreground system were derived from the in vivo trial (Rota Graziosi et al. submitted) concerning feed rations (ingredients and nutritive values) and animal performances (e.g., milk production, nitrogen balance). In particular, the daily milk production was pared to 32.7 kg/cow for CON diet and 33.2 kg/cow for SBS diet, the same productions were considered for the two scenarios (CON + RSM and SBS + RSM diets). Primary data were also collected by directly interviewing the farmer. Information about the cropping system (feed crops and their DM yields, tillage, methods adopted for feed conservation, fuel, purchased seeds, pesticides, and fertilizers), the purchased forages and concentrates (type and origin), the herd composition, and the manure management were collected, using a questionnaire.

Secondary data related to the background system (production of seeds, raw materials, fuels, fertilizers, pesticides, tractors and agricultural machines, transport) were obtained from the ECOINVENT® and the Agri-footprint databases.

Emission estimation

Feed emissions calculation. The environmental impact of feed raw materials was obtained from the Ecoinvent (2013), Eco-Alim (2015), and Agri-Footprint (2014) databases. The environmental impact of forages was calculated considering inputs needed at the field level (e.g., fossil fuel, seeds, fertilizers, pesticides, agricultural machines), feed processing (e.g., drying, ensiling), and transport.

The effects on direct and indirect N_2O emissions derived by the application on the field of organic (solid and slurry) and inorganic fertilizers, as well as crop residues, were accounted for, using Intergovernmental Panel on Climate Change guidelines (IPCC 2019b). Also, NO_3 emissions from organic and inorganic fertilizers application were considered (IPCC 2019b). NH_3 from manure and chemical fertilizers spreading was accounted for, using the European Environment Agency method (EEA 2019a; 2019b), as well as NO_2 from chemical fertilizers spread in the field (EEA 2019a). PO_4 transport to water resulting from chemical fertilizers spreading were computed as proposed by Nemecek and Kägi (2007).

For conventional soybean meal and oil, direct LUC was included in the assessment. Different LUC methods result in significantly different outputs; in this study, we used values reported by the Agri-Footprint database (Soybean meal, from crushing (solvent), at plant/BR Economic and Crude soybean oil, from crushing (solvent), at plant/BR Economic, Agri-Footprint, 2014). Therefore, 4.05 kg of CO_2eq/kg soybean meal and 11.2 kg of CO_2eq/kg soybean oil, for soybean from South America were considered. For all purchased conventional soybean meal and oil, an amount of 20% from Italy and 80% from South America was considered (ASSALZOO 2018). Responsible soybean meal and oil environmental impacts were evaluated based on data reported by Agri-Footprint database (Agri-Footprint 2020). The process of responsible soybean, at farm, describes the cultivation process of Soybeans in Brazil, from 14 participating farms in the ProAgros project (part of the Sustainable Farming Assurance Programme, SFAP). No LUC contributes to the environmental impact of the responsible soybean process. An economical allocation was performed for responsible soybeans at the farm (from SFAP farms), responsible soybean meal (solvent), responsible soybean hull (solvent) and responsible crude soybean oil (solvent). For all purchased responsible soybean meal and oil, we considered an amount of 20% from Italy and 80% from Brazil (i.e., responsible soy).

GHG emissions on farm. Table 3 shows the models used for on-farm GHG estimation. CH_4 emissions from livestock enteric fermentation were estimated using different equations: the equation from Niu et al. (2018) for lactating cows, while, for the other livestock categories, the equation of IPCC (2019a) was used. For lactating cows, DMI was derived by the in vivo trial of the companion paper (Rota Graziosi et al. submitted). CH_4 emissions from manure storage were estimated using the method suggested by the IPCC (2019a). Volatile solid excretion was estimated considering the gross energy of the diets (kJ/kg of DM) evaluated using the equation of Ewan (1989). Digestibility of the feed was estimated using a calculation model developed for each type of forage and concentrate feed on the basis of the equations proposed by INRA (2007). In vivo data about the chemical composition of feed and diets and digestibility, collected during the trial reported in the companion paper (Rota Graziosi et al. submitted), were used for lactating cow rations. N_2O emissions from manure storages occurred in direct and indirect forms and they were estimated using the method from IPCC (2019a). In the current study, animal nitrogen excretion of lactating cows was measured in vivo during the trial reported in the companion paper (Rota Graziosi et al. submitted). CO_2 emissions occurring during field operations (i.e., ploughing, harrowing, sowing, harvesting, and so on) were estimated using the processes of the Ecoinvent (2007) database. Emissions from livestock respiration and the variation in soil carbon stocks were not accounted for.

Other emissions on farm. Table 4 reports the models used for the estimation of acidifying and eutrophic substances emitted on farm. NH_3 and NO_2 emissions that occur during animal housing, manure storage, and spreading were estimated following the method proposed by EEA (2019a) and EEA (2019b), on the basis of the total amount of nitrogen excreted by the animals. Nitrogen excretion of lactating cows was measured in vivo (Rota Graziosi et al. submitted).

The NH_3-N and NO_2-N emission factors, as a proportion of total ammonia nitrogen, were specific for each manure type (slurry or solid) and each step in manure handling (EEA 2019b). The NH_3 and NO_2 emitted during manure spreading and application of synthetic fertilizers were estimated following EEA (2019a). The amount of N leached as NO_3 was estimated on the basis of N leached, following the IPCC (2019b) model. The amount of P lost in dissolved form to surface water (run-off) and leached was considered to estimate the transport to water of PO_4 as proposed by Nemecek and Kägi (2007).

Off farm processes emission. The emissions related to off farm activities were calculated using LCA software, Simapro PhD 7.3.3 (PRé Consultants 2012). The processes considered included the production chain of commercial feed (from crop growing to feed factory processing), production of purchased forages and bedding material, production of chemical fertilizers, pesticides, diesel, and electricity used in the farms. Transportation was accounted for feed and bedding materials.

Life cycle impact assessment (LCIA)

After classification, environmental impact was calculated using the characterization factors of ReCiPe Midpoint (H) V1.10 / Europe Recipe H. Normalization was also performed for milk production of the different scenarios through ReCiPe Midpoint (H) V1.10 / Europe Recipe H. The LCIA was performed using the SimaPro V 8.3 software tool.

Results and discussion

Environmental impact of soybean silage compared with other forage sources

Results obtained in the present study highlight differences, in terms of environmental impact, between soybean silage and the main farm-produced forages included in lactating cow diets (Table 5). For all the forages, the main contribution to global warming potential (GWP) was cultivation phases (especially GHG emissions into the air), followed by processing and transport, according to Mogensen et al. (2014). Soybean silage was more sustainable for GWP than barley silage and high moisture ear maize; the latter showed the highest value of GWP and HT (Table 5). The high water and N requirements of high moisture ear maize contributed significantly to GWP; this is consistent with Ma et al. (2012) in field experimental study on maize. Unlike maize, soybean silage is a low input crop regarding water and N fertilization (both organic and inorganic), which should be favourable in terms of GWP. Ma et al. (2012) reported that low N application decreases both total GHG emissions and the GWP across all the rotation systems; hence, a forage crop with low fertilization requirements is advisable to enhance the sustainability of the farming system. Barley Silage has a higher GWP than soybean silage (Table 5). The GWP value of barley was higher than the results of Mogensen et al. (2014), 285 kg CO_2eq/ton DM. In this latter work, differently than the present study, the authors did not take into account manure to the field but considered all fodder crops fertilized only by inorganic N. In González-García et al. (2016), in a study conducted in Spain, barley silage gave a value of GWP closer to the one of Mogensen et al. (2014) (i.e., 321 kg CO_2eq/ton DM), but the authors concluded that this value is highly dependent on agricultural practices and system boundaries. Hence a certain variation has to be expected across studies. Soybean silage showed a higher GWP value than maize silage, Italian ryegrass hay, and lucerne hay (Table 5**).** The latter is similar to soybean silage for DM yield and CP content, but showed a lower GWP, mainly due to the low contribution from field operation, in particular tillage. However, the large seeding window of soybean allows it to grow in succession to different winter crops, maximizing yields of DM and CP per hectare. For example, cultivating soybean for silage after a mixture of winter cereal and legume forages makes it possible to harvest more than 15 t DM/ha per year (Tabacco and Comino 2019). GWP obtained for lucerne hay was similar to the one reported by Adom et al. (2012), 170 kg CO_2eq/ton DM, but lower than the one reported by Fathollahi et al. (2018). This previous study found a value for the GWP of maize silage (329 CO_2eq/ton DM) similar to the present study. On the opposite, GWP of maize silage was slightly higher compared to the result obtained by Xu et al. (2018), 680 kg CO_2eq/ton AF, as well as to the data of Adom et al. (2012) and Mogensen et al. (2014), 200 and 224 kg CO_2eq/ton DM, respectively. Compared to the studies mentioned above, the difference can be explained by the organic fertilization of the maize silage of the present study and subsequent emissions into the air.

High human toxicity (HT) characterized high moisture ear maize, similarly to soybean silage and maize and barley silage (Table 5). This was mainly due to mechanical operations in the field (e.g., ensiling), besides the use of inorganic and organic fertilizers.

High values of TA and MA also characterized barley silage and high moisture ear maize, and this is correlated to the transport of nutrients to groundwater which is linked to the consistent use of manure adopted by the present farm. Compared to the barley of González-García et al. (2016), and to the corn silage and alfalfa hay of Fathollahi et al. (2018), TA was higher in the present study for barley (23.9 vs. 14.1 kg SO_2eq/ton DM), corn (15.6 vs. 7.1), but lower for alfalfa (0.57 vs. 5.81). This was probably due to higher manure application. In the present study, most solid manure produced by cows was spread on annual crops for ensiling, excluding soybean, to reduce the growing period and favour high yield at harvest. Soybean silage and alfalfa hay had rather low results of TA and ME because organic and inorganic fertilizers were not used.

The highest agricultural land occupation (ALO) was observed for soybean silage (Table 5). This impact category follows the phase of land transformation from natural to human utilization; the occupation affects the original habitat and the original species composition (Huijbregts et al. 2016). The high value of ALO reported for soybean silage was probably due to the wide use of herbicides and insecticides, especially compared with the other forages. For example, barley silage, lucerne hay, and Italian ryegrass hay, which did not require the use of pesticides, had similar values for ALO. Soybean silage also showed the highest result for natural land transformation (NLT), similar to maize crops and barley silage (Table 5). NLT in Fathollahi et al. (2018) was 0.07 and 0.08 m^2 for corn silage and alfalfa hay, respectively, lower than the present study.

Since soybean silage is an important contributor to the protein requirement of lactating cows, its sustainability for GWP and ME was compared with that of other forage sources in terms of CP yield (Figure 2). Soybean silage protein yield (t/ha) was similar to lucerne hay (Table 1), even though the GWP per unit of CP of soybean silage was twice that of lucerne hay (2439 and 1034 kg CO_2eq/ton CP, respectively). However, being an annual crop, soybean has some agronomic and management advantages compared to lucerne, as it is easier to insert in crop rotation with maize and it gives farmers more opportunities to apply manure; in addition, the same machinery used to plant and harvest maize can be used for soybean (Seiter et al. 2004). Moreover, the large seeding window of soybean silage allows maximizing yields of CP/ha and the succession to winter crops. Also for ME, the environmental impact of soybean silage was higher than lucerne hay, i.e. 25 and 13 kg Neq/ton CP, respectively. Overall, the results of GWP and ME were greatly influenced by the CP content: the greater the content, the lower the impact with the exception for maize silage for which the high amount of biomass leads to high CP yield (ton CP/ha, Table 1) and consequently to lower environmental impact, in terms of CP, compared to the other forage sources (i.e., barley silage, high moisture ear maize, and Italian ryegrass).

Global warming potential of lactating cow diets based on different soybean sources

The GWP of individual daily TMR was calculated as the sum of GWP of each feed ingredient; the average value was pared to 17.8 kg CO_2eq, similar to the results reported by Gislon et al. (2020a), i.e. 13.7 kg of CO_2eq, with a wide variation among farms. As suggested by these authors, the variability of GWP among diets is directly related to their feed composition and to the GWP of each feed. In particular, there is a linear correspondence between increasing daily diet GWP (kg of CO_2eq) and increasing the amount of soybean meal in the ration (Gislon et al. 2020a). This mainly explained the higher values of GWP obtained in the present study for CON and SBS diets as compared to diets including RSM, mainly due to LUC of conventional soybean meal. The impact of soybean meal on the total diet GWP was 43% and 31% for CON and SBS, where part of the soybean meal was replaced by soybean silage. On the contrary, the impact of soybean meal, coming from responsible soy cultivation, contributed only for the 11% and 7% to the GWP of CON + RSM and SBS + RSM diets, respectively (Figure 3). The impact of soybean meal in the four diets considered was 9.81, 6.34, 1.48, 0.96 kg CO_2eq, for CON, SBS, CON + RSM, and SBS + RSM diets, respectively (Figure 3). Therefore, the highest value of individually daily diet GWP was observed with the CON diet (23.0 kg CO_2eq), but this value was reduced when the soybean meal was substituted either with the responsible soybean meal (CON + RSM, 13.4 kg CO_2eq) or soybean silage (SBS, 20.8 kg CO_2eq, Figure 3).

For all four diets, maize (forages and concentrates) gave an important contribution to the GWP by providing, on the whole, about 7 kg CO_2eq (Figure 3). In this regard, it is important to apply crop management strategies that can lower the GWP. For example, as Adom et al. (2012) suggested, fertilizer best management practices such as precision application of farm nutrients may significantly reduce maize GWP. Despite the high environmental impact of high moisture ear maize (Table 5), SBS diet, characterized by a higher inclusion of this feed than CON, showed lower GWP than CON. Therefore, the partial replacement of conventional soybean meal with soybean silage, even combined with the inclusion in the diet of high moisture ear maize, gave an interesting result in reducing global daily diet GWP. These results, therefore, encourage the inclusion of these soybean sources into lactating cow diets rather than conventional soybean meals. In addition, the partial replacement in the diet of maize silage with high moisture ear maize allowed to reduce the inclusion in the diet of maize meal (Table 2). This may be favourable in terms of daily diet GWP since reduced dietary concentrates might reduce total net emissions (Ogino et al. 2007). Furthermore, excessive use of maize meal in the diet is not related to any productive advantage for the animals (Gislon et al. 2020a).

Environmental impact of milk production on the basis of different scenarios

The dietary formulation is an interesting way to reduce the GWP of diets for dairy cows and, as a consequence, the overall environmental impact of milk production (Wilkinson and Garnsworthy 2017). Following this suggestion, the environmental impact of milk from animals fed diets based on different sources of soybean was evaluated (Table 6). The partial replacement of conventional soybean meal with soybean silage and the total replacement of conventional soybean meal with responsible soybean meal allowed the reduction of the environmental impact of milk production for all the categories studied (Table 6). Therefore, the GWP of milk decreased from 1.38 kg CO_2eq/kg FPCM of the CON scenario to 1.17 and 1.13 kg CO_2eq/kg FPCM of the SBS and CON + RSM. In terms of GWP, the best result was reached by combining soybean silage with responsible soybean meal: 1.01 kg CO_2eq/kg FPCM (Table 6). Overall there was a wide variation of GWP among scenarios, despite the similar milk production. This variation for GWP per kg of FPCM was also observed by Battini et al. (2016), analysing 4 dairy farms in the Po Valley: the values ranged from 1.18 to 1.60 kg CO_2eq/kg FPCM, when LUC and C sequestration were not considered, and from 1.56 to 1.89 kg CO_2eq/kg FPCM when they were considered. In the study of Uddin et al. (2020), two diets with an inclusion of soybean meal similar to CON (i.e., 11.9% on average) had also similar GWP with CON: 1.44 CO_2eq/kg FPCM, on average (Uddin et al. 2021).

For those scenarios involving conventional soybean meal, CON and SBS, even if partially replaced with soybean silage for the latter, feed production, on farm and off farm, accounted for 47% (CON) and 42% (SBS) of milk GWP, even slightly higher than the contribution given by animal housing, i.e. 30% and 32% of total GWP. By replacing conventional soybean with responsible soy (CON + RSM and SBS + RSM scenarios), the GWP contribution from feed production decreased to 35%, with a share related to animal housing of 37% of milk GWP.

Values of GWP obtained for CON and SBS scenarios are mainly linked to purchased protein sources, particularly soybean and LUC. The LUC is identified (Castanheira and Freire 2013) as the main source of GHG emissions from this crop. Thus, increasing farm protein self-sufficiency by producing high quality forages, such as soybean silage, may increase the environmental sustainability of the milk chain. These findings were confirmed in March et al. (2021), where the scenario with a diet based on home-grown forages, in particular with legume beans and silages as protein sources (no soybean meal included), was the one with lower GWP (1.18 kg CO_2eq/kg FPCM). Otherwise, reducing the reliance on imported soybean meal in EU would require deep changes in dietary patterns, crop and livestock production, and world trading (Karlsson et al. 2021).

Besides the favourable use of soybean silage, the present study demonstrated the great potential of responsible soybean meal to increase the sustainability of milk production (Table 6). Results obtained for NLT were also influenced by conventional soybean included in the scenarios, showing lower values as this is reduced (Table 6). Results are consistent with Vagnoni and Franca (2018), highlighting that diets characterized by larger use of soybean-based feed result in higher emissions related to the land transformation from the forest. This is also confirmed by Mueller et al. (2014), showing a close relationship between land transformation and soybean meal for intensive milk production systems.

HT was mainly related to the feed production, both on farm and off farm, for all the scenarios considered (Table 6). The slight differences detected among the scenarios were mainly due to emissions related to purchased feed. In particular, a greater contribution from maize meal emerged, which, in fact, was almost halved in SBS and SBS + RSM scenarios (Table 2), showing the lowest values for HT (Table 6).

No differences occurred in terms of TA and ME, among the scenarios considered (Table 6). This is mainly related to the fact that these impact categories are mainly linked to manure management. In the present study it was assumed that the same mode of animal housing, manure storage, and spreading were implemented at the farm for all the scenarios.

Data normalization

A normalization of the data was carried out with Recipe Midpoint (H) using European normalization references (the average European inhabitant environmental load, for each impact category, Figure 4). The normalization step provides adimensional scores, useful to understand the relative importance of category indicator results for a single product system (Guinée et al. 2002). Results obtained from normalization allow identifying possible improvements in the environmental performance of milk production since it addresses the activities of major contributors to environmental impact. According to these outcomes, the impact category that can be regarded as highly significant is NLT, regarding CON and SBS scenarios, due to the utilization of conventional soybean. Total substitution of soybean meal with soybean silage and responsible soy (SBS + RSM and CON + RSM) reduced the impact of NLT (Figure 4).

The other significant impact categories were TA and ME, for all the scenarios considered, consistent with the results of Hospido et al. (2003). Since crop production for animal feed is responsible for an important percentage of several impact categories, such as ME and TA (Hospido et al. 2003), some alternatives to reduce the environmental impact of milk production can be proposed. Increasing efficiency of forage production and use are examples of sustainable intensification and contribute to improve the environmental sustainability of milk production (Gislon et al. 2020a).

ALO, GWP and HT can be classified as impact categories that did not have a significant effect (Figure 4).

Conclusion

The high environmental impact of imported soybean meal mainly due to intensive and destructive use of land in the country of origin creates the urgency to find alternative feed ingredients. An alternative option is the inclusion of soybean silage into lactating cow rations. The use of soybean silage contributed to a reduced GWP of the daily diet and the environmental impact of milk production, due to the reduction of soybean meal inclusion. However, compared with lucerne hay, the most utilized self-produced protein feed in Italian dairy farms, soybean silage showed higher GWP, ME and HT mainly due to the high contribution of mechanical operations in the field (e.g., tillage).

In addition, the substitution of conventional soybean meal with responsible soybean allows the opportunity to achieve high sustainability of milk production when considering GWP, ALO, NLT, and HT. The normalization of impact categories highlights the negative effect of conventional soybean meal on NLT and puts in evidence the positive effect of the inclusion in lactating cow diets of responsible soybean.

In conclusion, the use of soybean silage is an interesting option to reduce environmental impact of milk production besides maximizing yields of DM and CP per hectare if grown in succession to different winter crops and be able to be grown instead of lucerne. Responsible soybean meal resulted to be another interesting protein feed choice to increase sustainability of milk chain.

Disclosure statement

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

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author, G.G., upon reasonable request.

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Table 1. Characteristics of soybean silage compared to other forage sources.

Crop Period	**Crop**	**Feed**	**UAA[1]**	**ton AF[2]/ha**	**ton AF**	**ton DM[3]/ha**	**% DM**	**ton CP[4]/ha**	**CP %DM**
Spring-Summer	Soybean	Soybean silage	16	33.0	528	9.2	28.0	1.8	19.5
Spring-Summer	Maize	High moisture ear maize	10	19.5	195	12.9	66.0	1.1	8.4
Spring-Summer	Maize	Maize silage	10	55.0	550	19.3	35.0	1.5	8.0
Plurennial	Lucerne	Lucerne hay	11.5	12.0	138	10.3	86.0	2.0	19.0
Autumn-Winter	Barley	Barley silage	16	35.3	565	11.3	32.0	1.2	11.0
Autumn-Winter	Italian Ryegrass	Italian ryegrass hay	11	12.0	132	10.3	86.0	0.8	8.0

[1] UAA= utilised agricultural area

[2] AF=as fed

[3] DM= dry matter

[4] CP= crude protein

Table 2. Ingredients and chemical composition of the four lactating cows rations.

Item (% of DM)	**CON[1]**	**SBS[2]**	**CON + RSM[3]**	**SBS + RSM[4]**
High moisture ear maize	-	15.6	-	15.6
Soybean silage	-	12.4	-	12.4
Barley silage	10.3	10.2	10.3	10.23
Lucerne hay	9.52	9.43	9.52	9.43
Maize silage	25.5	9.22	25.5	9.22
Maize meal	16.4	8.72	16.4	8.72
Flaked maize grain	7.31	7.27	7.31	7.27
Soybean meal	10.7	6.91	-	-
Responsible soybean meal	-	-	10.7	6.91
Molasses cane	4.83	4.81	4.83	4.81
Wheat straw	1.95	1.95	1.95	1.95
Sunflower meal	5.00	4.94	5.00	4.94
Barley grain	2.80	2.79	2.80	2.79
Maize gluten feed	1.50	1.51	1.50	1.51
Wheat shorts	2.30	2.25	2.30	2.25
Soybean oil	0.60	0.62	-	-
Responsible soybean oil	-	-	0.60	0.62
MinVit[5]	1.30	1.32	1.30	1.32
Chemical composition (as % of DM unless differently stated)				
DM (% of AF)	49.6	48.9	49.6	48.9
Ash	5.90	6.38	5.90	6.38
OM	94.1	93.6	94.1	93.6
CP	14.7	15.2	14.7	15.2
Sol CP (% of CP)	21.6	29.8	21.6	29.8
EE	2.72	3.33	2.72	3.33
NDF	30.6	30.4	30.6	30.4
ADF	18.8	18.9	18.8	18.9
ADL	3.81	3.89	3.81	3.89
NDIP	1.32	1.34	1.32	1.34
ADIP	1.03	0.96	1.03	0.96
NFC	46.0	44.8	46.0	44.8
N-NH_3 (% of total N)	15.1	13.9	15.1	13.9

[1] CON= conventional soybean meal

[2] SBS= conventional soybean meal and soybean silage

[3] CON + RSM= responsible soybean meal

[4] SBS + RSM= soybean silage and responsible soybean meal

[5] Each kg contained: 31 g Fe, 70.5 g Zn, 30.4 g Mn, 100 mg Se, 2 g I, 60 mg Mo, 6.9 g Cu, 500 mg beta carotene, 4,000,000 IU Vitamin A, 800,000 IU Vitamin D3, 20,500 IU Vitamin E, 2450 IU Vitamin B1, 343 IU Vitamin B6, 20 IU Vitamin B12, and 52,000 IU Vitamin PP

Table 3. Equations used for the estimation of the GHG emissions on farm.

Pollutant	**Source**	**Equation**	**Reference**
CH_4	**Enteric**	Lactating cows: CH_4 (kg head^{-1} yr^{-1})= $((124 + 13.3 \cdot DMI)) \cdot 365/1000$	Equation 2 in Niu (2018)
		Others: CH_4 (kg head^{-1} yr^{-1})= $(GE^{1} \cdot (Ym/100))/55.65$	Equation 10.21 in IPCC (2019a)
		GE (kJ) = $17{,}350 + (234.46 \cdot EE\%^{2}) + (62.8 \cdot CP\%) - (184.22 \cdot Ash\ \%)$	Ewan (1989)
		Ym= 6.3	Table 10.12 in IPCC (2019a)
	Manure storage	CH_4 (kg head^{-1} yr^{-1}) = $(VS^{3} \cdot 365) \times [B0^{4} \cdot 0.67 \cdot MCF^{5}/100 \cdot AWMS^{6}]$	Equation 10.23 in IPCC (2019a)
		VS (kg day^{-1})= $[GE \cdot (1 - DE^{7}/100) + (UE \cdot GE^{8})] \cdot [(1 - Ash)/18.45]$	Equation 10.24 in IPCC (2019a)
		DE: feed digestibility	INRA (2007)
		B0 dairy cattle: 0.24	Table 10.16 in IPCC (2019a)
		B0 non dairy cattle: 0.18	Table 10.16 in IPCC (2019a)
		MCF liquid slurry and pit storage: 37%	Table 10.17 in IPCC (2019a)
		MCF solid storage: 4%	Table 10.17 in IPCC (2019a)
N_2O direct	**Manure storage**	N_2O (kg yr^{-1}) = $[\sum[\sum((N \cdot Nex^{9}) \cdot AWMS) + N] \cdot EF^{10}] \cdot 44/28$	Equation 10.25 in IPCC (2019a)
		N: annual nitrogen input via co-digestate in the country	
		EF solid storage: 0.01	Table 10.21 in IPCC (2019a)
		EF liquid slurry: 0.005	Table 10.21 in IPCC (2019a)
		EF pit storage: 0.002	Table 10.21 in IPCC (2019a)
	Field	$N_2O = (Fsn^{11} + Fon^{12}) \cdot EF + (Fcr^{13} + Fsom^{14}) \cdot EF \cdot 44/28$	Equation 11.2 in IPCC (2019b)
		Fon: annual amount of N from managed animal manure applied to soil ($Nex - Frac\_Loss^{15} + N\ bedding^{16}$)	Equation 10.34 in IPCC (2019a)
		Frac Loss= Frac Gas + Frac Leachs + Frac N_2MS + EF	Equation 10.34A in IPCC (2019a)
		EF solid storage: 0.01	Table 10.21 in IPCC (2019a)
		EF liquid slurry: 0.005	Table 10.21 in IPCC (2019a)
		EF pit storage: 0.002	Table 10.21 in IPCC (2019a)
N_2O indirect	**Manure storage**	N_2O = N volatilization$^{17} \cdot EF \cdot 44/28$	Equation 10.28 in IPCC (2019a)
		N volatilization= $((Nex \cdot AWMS) + Ncdg) \cdot Frac\ Gas\ \_MS^{18}/100$	Equation 10.26 in IPCC (2019a)
		Frac Gas _MS solid storage: 0.30	Table 10.22 in IPCC (2019a)
		Frac Gas _MS liquid slurry: 0.30	Table 10.22 in IPCC (2019a)
		Frac Gas _MS pit storage: 0.28	Table 10.22 in IPCC (2019a)
		EF: 0.01	Table 11.3 in IPCC (2019b)

Field	$N_2O(ATDN) = [(Fsn \cdot Frac\_GasF^{19}) + (Fon \cdot Frac\_GasM^{20})] \cdot EF \cdot 44/28$	Equation 11.9 in IPCC (2019b)
	Frac_GasF: 0.11	Table 11.3 in IPCC (2019b)
	Frac_GasM: 0.21	Table 11.3 in IPCC (2019b)
	EF: 0.01	Table 11.3 in IPCC (2019b)
	$N_2O(L) = (Fsn + Fon + Fprp + Fcr + Fsom) \cdot Frac\_Leachs^{21} \cdot EF \cdot 44/28$	Equation 11.10 in IPCC (2019b)
	Frac_Leach: 0.24	Table 11.3 in IPCC (2019b)
	EF: 0.011	Table 11.3 in IPCC (2019b)

[1]GE = gross energy intake (MJ/ d); [2]EE% = ether extract of feed (% DM); [3]VS = daily volatile solid excreted (kg of DM /animal); [4]B0 = maximum methane-producing capacity for manure (m^3); [5]MCF = methane conversion factors for each given manure management system (%); [6]AWMS = fraction of livestock manure handled using each given manure management system (dimensionless); [7]DE% =energy digestibility of feed (%); [8](UE · GE) =urinary energy expressed as fraction of GE (dimensionless); [9]Nex = annual N excretion (kg of N/animal); [10]EF = emission factor for direct N_2O emissions from a given manure management system (kg of N_2O-N/kg of N in manure management system); [11]Fsn = annual amount of synthetic fertilizer N applied to soils (kg of N); [12]Fon = annual amount of animal manure, compost, sewage sludge and other organic N additions applied to soils (kg of N); [13]Fcr= annual amount of N in crop residues (above and below ground), including N-fixing crops, and from forage/pasture renewal, returned to soils (kg of N); [14]Fsom= amount of N mineralised from loss in soil organic C in mineral soils through land-use change or management practices; [15]Frac_loss = fraction of managed manure N that is lost in a given manure management system (%); [16]N_bedding = annual amount of N from bedding (kg of N/animal); [17]N volatilization = annual amount of manure N that is lost due to volatilization of NH_3 and nitric oxide compounds (NO_x; kg of N); [18]Frac_GasMS = fraction of managed manure N that volatilizes as NH_3 and NO_x in a given manure management system (%) ; [19]Frac_GasF = fraction of synthetic fertilizer N that volatilizes as NH_3 and NO_x (%); [20]Frac_GasM = fraction of applied organic N fertilizer materials and of urine and dung N deposited by grazing animals that volatilizes as NH_3 and NO_x (%); [21]Frac_Leach = N fraction lost through leaching and runoff (%).

Table 4. Equations for the estimation of non-GHG emissions on farm.

Pollutant	Source	Equation	Reference
NH_3	**Housing**	TAN[1] = Nex[2] · EF_TAN[3]	Equation 10 in EEA (2019a)
		EF_TAN: 0.6	Table 3.9 in EEA (2019a)
		NH_3hous_slurry = TAN hous_slurry · EF hous_slurry · 17/14	Equation 15 in EEA (2019a)
		EF hous_slurry[4]: 0.24	Table 3.9 in EEA (2019a)
		NH_3hous_solid = TAN hous_solid · EF hous_solid · 17/14	Equation 16 in EEA (2019a)
		EF hous_solid[5]: 0.08	Table 3.9 in EEA (2019a)
	Manure storage	NH_3storage_solid = TAN storage_slurry · EF storage_slurry · 17/14	Equation 33 in EEA (2019a)
		EF storage_slurry[6]: 0.25	Table 3.9 in EEA (2019a)
		NH_3storage_solid = TAN storage_solid · EF storage_solid · 17/14	Equation 34 in EEA (2019a)
		EF storage_solid[7]: 0.32	Table 3.9 in EEA (2019a)
	Field	NH_3applic_slurry = TAN slurry_applic · EFapplic_slurry · 17/14	Equation 39 in EEA (2019a)
		EF applic_slurry[8]: 0.55	Table 3.9 in EEA (2019a)
		NH_3applic_solid = TAN solid_applic · EF applic_solid · 17/14	Equation 40 in EEA (2019a)
		EF applic_solid[9]: 0.68	Table 3.9 in EEA (2019a)
		NH_3applic_fert[10] = N fert_applic[11] · EF fert_type[12]	Equation 4 in EEA (2019b)
		EF urea: 159	Table 3.2 in EEA (2019b)
		EF amm.nitr[13]: 0.016	Table 3.2 in EEA (2019b)
		EF NPK[14]: 0.067	Table 3.2 in EEA (2019b)
		EF other straight N compounds: 0.014	Table 3.2 in EEA (2019b)
NO_2	**Manure storage**	EMMS_NO_2[15] = (Estorage_NO_slurry + Estorage_NO_solid) · 46/14	Equation 47 in EEA (2019a)
	Field	NO_2applic_tot = (Nslurry_applic + Nsolid_applic + Nfert_applic) · EFapplic	
		EFapplic: 0.04	Table 3.1 in EEA (2019b)
NO_3	**Field**	NO_3= N_leached · 4.426	
		N_leached=(Fsn + Fon + Fprp + Fcr + Fsom) · Frac_Leach	Equation 11.10 in IPCC (2019b)
		Frac_Leach: 0.24	Table 11.3 in IPCC (2019b)
PO_4^{3-}	**Field**	Pgw (leached to ground water)[16] = Pgwl[17] · Fgw[18]	Paragraph 4.4.3 in Nemecek and Kägi (2007)
		Pgwl arable land: 0.07	
		Pgwl permanent pasture and meadow: 0.06	
		Fgw: 1 + 0.2/80 · P_2O_5slurry	

Pro[19] (P lost through run-off to rivers) = Prol[20] · Fro[21]
Prol open arable land: 0.175
Prol extensive meadow: 0.25
Fro fert: 0.2/80 · P_2O_5fert
Fro slurry: 0.7/80 · P_2O_5slurry
Fro manure: 0.4/80 · P_2O_5manure

1TAN = total ammoniacal-N; 2Nex = annual average N excretion per head (kg of N/animal); 3EF_TAN = emission factor of TAN; 4hous_slurry = liquid slurry in the livestock buildings; 5hous_solid = solid manure in the livestock buildings; 6storage_slurry = liquid slurry in storages; 7storage_solid = solid manure in storages; 8applic_slurry = application of liquid slurry to the field; 9applic_solid = application of solid manure to the field; 10NH3 applic_fert =emission from fertilizer application to the field; 11N fert_applic = total N from fertilizer application; 12EF fert_type = emission factor for fertilizer type; 13Amm nitr = ammonium nitrate; 14NPK = nitrogen-phosphorus-potassium fertilizer; 15NO2 = nitrogen dioxide; 16Pgw = quantity of phosphorus leached to ground water (kg/ha); 17Pgwl = average quantity of phosphorus leached to ground water for each land use category (kg/ha); 18Fgw =correction factor for fertilization by slurry; 19Pro = quantity of phosphorus lost through runoff to rivers (kg/ha); 20Prol = average quantity of phosphorus lost through runoff to rivers for each land use category (kg/ha); 21Fro = correction factor for fertilization with each source of phosphorus.

Table 5. Environmental impact of soybean silage compared with that of other forage sources (ton DM).

Impact category	**Unit**	**Soybean silage**	**Maize silage**	**High moisture ear maize**	**Barley silage**	**Lucerne hay**	**Italian ryegrass hay**
Global warming potential (GWP)	kg CO_2eq	477	308	707	597	201	346
Terrestrial acidification (TA)	kg SO_2eq	0.39	15.6	23.2	23.9	0.57	13.3
Marine eutrophication (ME)	kg Neq	4.87	8.84	12.1	12.8	2.55	7.80
Agricultural land occupation (ALO)	m^2a	1105	533	787	902	986	996
Natural land transformation (NLT)	m^2	0.036	0.021	0.034	0.023	0.013	0.017
Human toxicity (HT)	kg 1,4-DBeq	10.97	13.69	17.50	13.29	9.30	9.93

Table 6. Environmental impact related to the milk production of different scenarios.

Impact category	Unit	CON[1]	SBS[2]	CON + RSM[3]	SBS + RSM[4]
Global warming potential (GWP)	kg CO_2eq	1.38	1.17	1.13	1.01
Terrestrial acidification (TA)	kg SO_2eq	0.023	0.022	0.022	0.021
Marine eutrophication (ME)	kg Neq	0.008	0.007	0.007	0.007
Agricultural land occupation (ALO)	m^2a	0.94	0.91	0.77	0.84
Natural land transformation (NLT)	m^2	0.00371	0.00252	0.00005	0.00004
Human toxicity (HT)	kg 1,4-DBeq	0.037	0.028	0.033	0.027

[1] CON= conventional soybean meal

[2] SBS= conventional soybean meal and soybean silage

[3] CON + RSM= responsible soybean meal

[4] SBS + RSM= soybean silage and responsible soybean meal

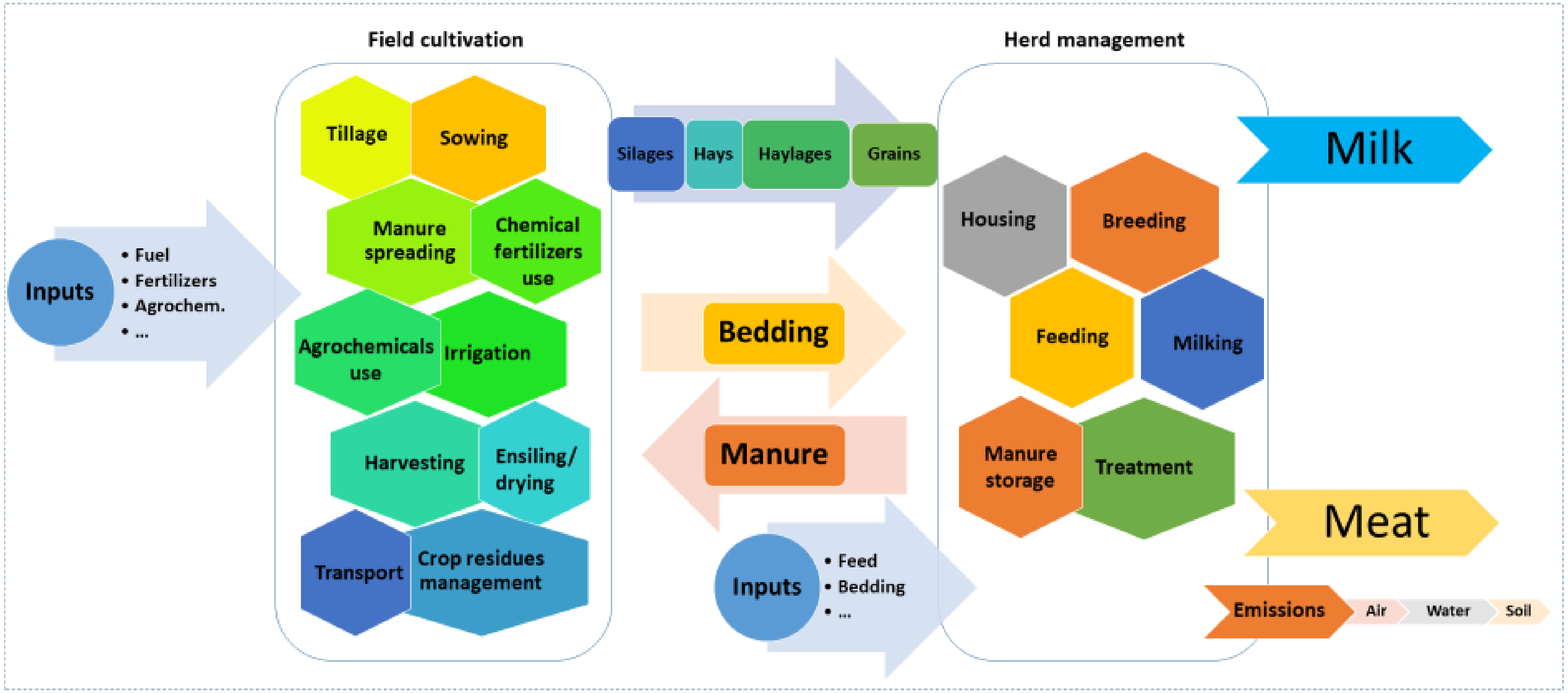


Figure 1. System boundaries.

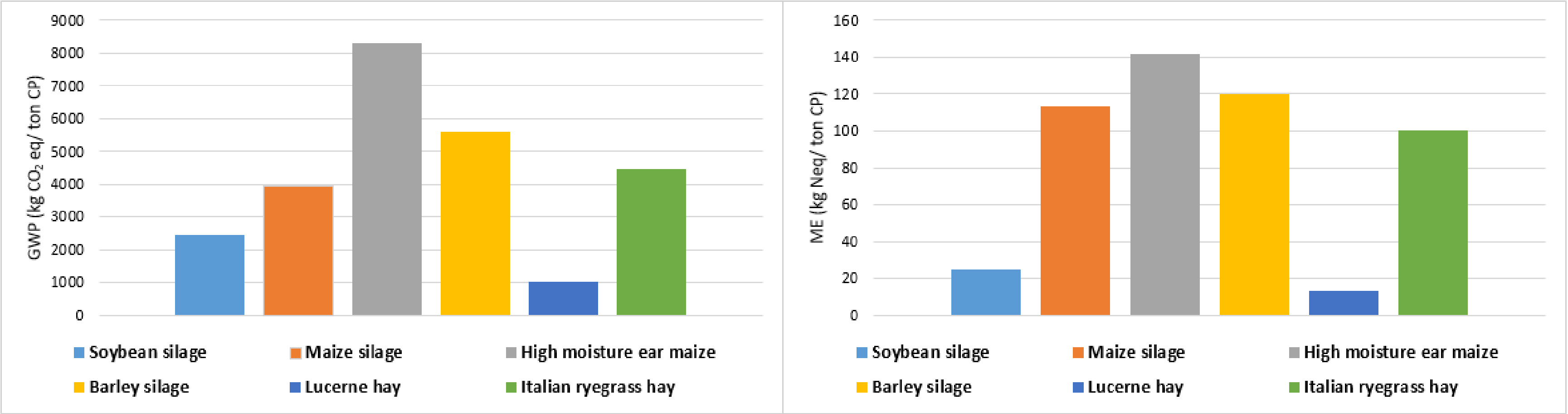
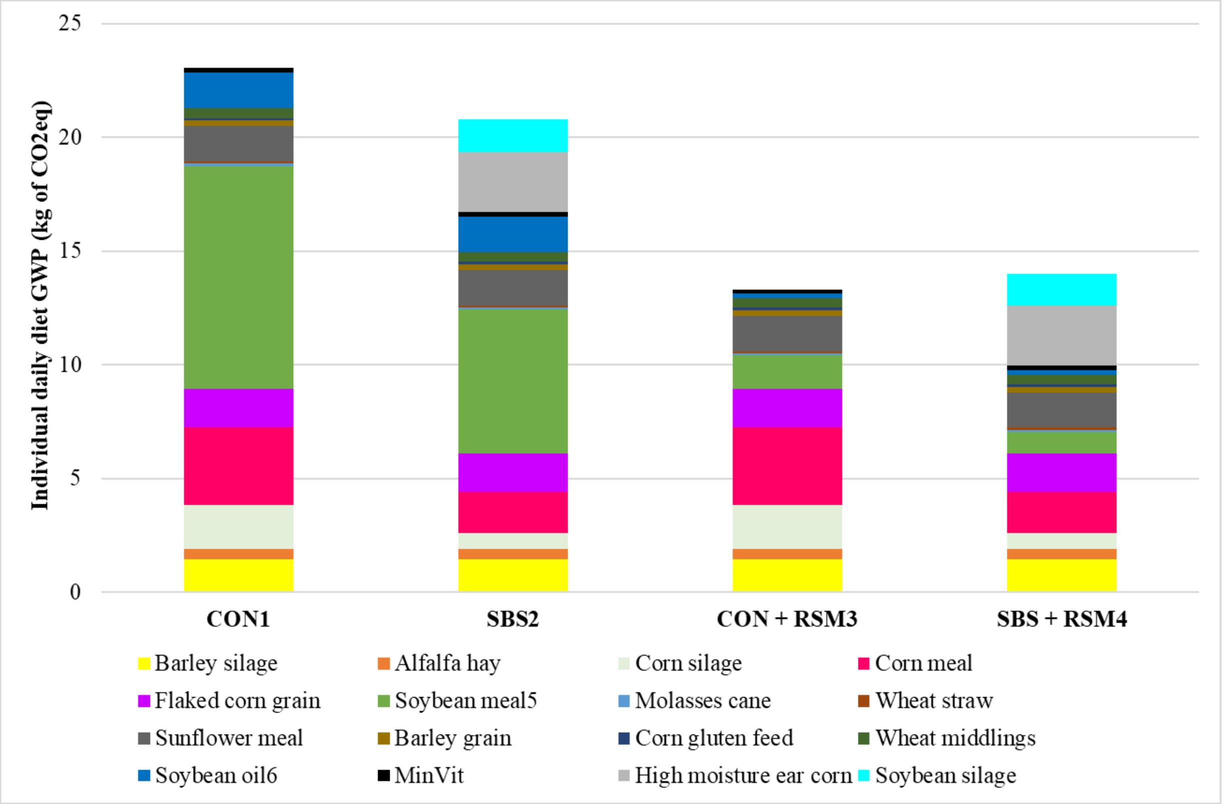


Figure 2. Environmental impact in terms of global warming potential (GWP) and marine eutrophication (ME) of soybean silage compared with that of other forage sources (ton CP).



[1] CON= conventional soybean meal

[2] SBS= conventional soybean meal and soybean silage

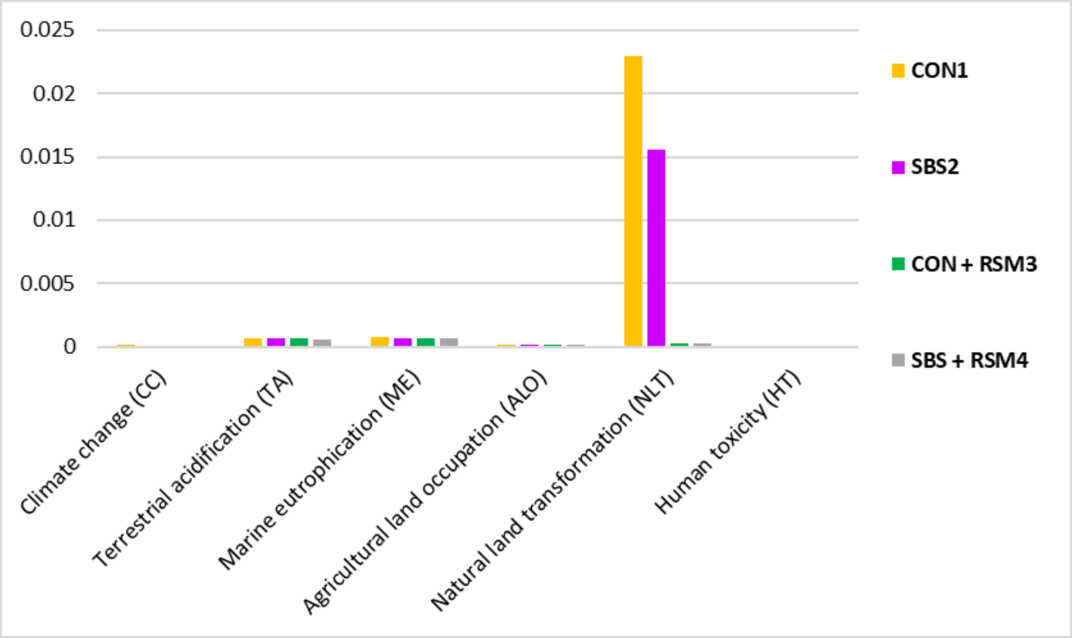
[3] CON + RSM= responsible soybean meal

[4] SBS + RSM= soybean silage and responsible soybean meal

[5] Soybean meal either conventional for CON and SBS or responsible for CON + RSM and SBS+RSM

[6] Soybean oil either conventional for CON and SBS or responsible for CON + RSM and SBS + RSM

Figure 3. Global warming potential (GWP) of individual daily lactating cows TMR, characterized by different soybean sources.



[1]CON= conventional soybean meal

[2]SBS= conventional soybean meal and soybean silage

[3]CON + RSM= responsible soybean meal

[4] SBS + RSM= soybean silage and responsible soybean meal

Figure 4. Normalisation data for each impact category considered in the present study.

8. General conclusions

Mitigation of the environmental impact of dairy cows through their nutrition is possible and advisable. As highlighted in the present PhD project, through diet management, it is possible to act on direct emission, like enteric CH_4, or emission per unit of product, enhancing cows' productivity. Moreover, indirect emissions, like CO_2 emission due to LUC, can also be addressed using more sustainable feed ingredients without impairing animal performance. In general, a more sustainable diet is also connected to other positive effects. The choice and use of feed additives can redirect rumen fermentation to be more functional to cow health and productivity, but the economic profit strongly depends on the price of the additive and the magnitude of the positive effect. The use of *Achillea moschata* EO (chapter 3) and its pure component as a feed additive was promising to reduce CH_4 emission although with different effects depending on the incubation time; however, further studies need to be conducted to understand its possible application in vivo. For example, some differences had been observed between the short and long incubation, potentially due to different experimental conditions (i.e. different doses used, different donor animals) or to the possible adaptation of rumen bacteria to the additives. In this latter case, further investigation would be required to understand the long-time effect of these additives. It has to be noticed that the in vitro steps are needed to evaluate the potential of some additives but, as underlined by the European Food Safety Authority, in vivo trials are mandatory for additive registration. The treatment affected the VFA composition, probably because of increased ruminal hydrogen availability due to methanogenesis inhibition, which might enhance the fermentation pathways that consume hydrogen, such as the formate, valerate, and caproate biosynthesis process. The role of the different rumen microbial populations in the CH_4 emission was confirmed in this study with a negative relationship between Proteobacteria and methane production. A higher abundance of Proteobacteria in low-CH_4 emitting cows than in high-CH_4 emitting ones was found in other studies, in agreement with what was found with the addition of EUCA and CAM in the short incubation trial. Furthermore, the total number of protozoa was markedly increased (i.e. 30-50%) by the treatments in both experiments (short and long incubation). This result was unexpected given the reduction obtained for CH_4 production and the assumed positive role of protozoa in rumen methanogenesis. If confirmed, the positive effect of *Achillea moschata* EO and its pure compounds on rumen protozoa could be exploited to investigate the role of these microorganisms in rumen metabolism since it was not well defined yet and contrasting results are observed in the literature.

Feed additives are included in small concentration into dairy cow diets. The highest percentage of diet DM is typically represented by forages. As explained above, they could also be managed to directly mitigate CH_4 emission. Forages are crucial, within a commercial dairy farm, because they affect the economic, productive, and environmental sustainability of the farm. The forage system could be a means to maintain farm competitiveness, increase feed self-sufficiency and integrating more livestock and vegetal production system. The aim should be to satisfy the requirements of high-producing dairy cows, providing feedstuff of high quality (i.e. highly digestible and with high nutrient density) produced in the farm. On the other hand, the forage crops might also improve the environmental sustainability of the farm. Optimizing the forages grown on the farm and reducing the reliance on protein sources bought on the market can lead to agronomic (i.e. legume forages) and economic advantages due to lower exposure to price volatility.

The meta-analysis (chapter 4) provided information on a wide dataset on the effect of each forage on productivity and enteric CH_4 emission. In the literature, different meta-analysis addressed the mitigation of enteric CH_4 emission in vivo, but the focus was on feed additives like nitrate, 3-nitrooxypropanol, monensin, lipids, or EO. The novelty of this study was that the main focus was the forage basis of the diet, as mitigation strategy. These indications could be used as another criterion to choose the best forages to be fed. The chemical composition (i.e. DM, OM, NDF, starch, and CP) and the NDFD of the diets were affected by the main forage included. Production performances and DMI were also affected. These differences partly explained the different results obtained for CH_4 emission, yield, and intensity. Corn silage and alfalfa silage diets had higher intake levels than grass silage and green forage, probably due to faster fermentation and physical breakdown in the rumen. Consequently, cows fed these two diets had higher milk production, confirming the high correlation between DMI and milk yield in this dataset. The choice of the forage basis of the diet was important in determining differences in CH_4 emission per kg of DMI or milk yield: corn silage diet had lower CH_4 yield than grass silage diets and lower CH_4 intensity than grass silage and green forage diets. Corn silage-based and alfalfa-based diets did not differ in terms of CH_4 emission, yield, and intensity. An important factor that might have determined the different values of DMI, milk production, and CH_4 emission might be starch degradation in the rumen, higher for corn silage diets. Starch fermentation in the rumen favors propionate production rather than acetate, reducing the hydrogen available for CH_4 synthesis, but it can also reduce NDFD when pH decreases too sharply. Thus, diet with excessive inclusion of starchy concentrates could reduce the exploitation from cows of human nonedible feedstuffs (rich in NDF), like forages, while providing excessive amount of potentially human edible feeds. This would further increase the competition for these limited resources.

Corn silage proved to be a forage basis sustaining high production and low CH_4 emission per kg of DMI or milk yield, probably due to its high energy density. However, even considering its high yield per hectare (in terms of DM or energy), the mono-cropping of this forage is not advisable. Instead, multi-annual/perennial forages (like pasture and grassland, either grazed or harvested, or alfalfa) had advantages compared to corn silage, like increasing soil carbon that is another GHG mitigation strategy. Other environmental benefits connected to these forages would be better evaluated through an LCA approach.

The meta-analysis was also conducted because it might be useful to find proxies highly correlated with CH_4 emission but easier to measure on a large scale and with less money and labor costs. Diet chemical composition provided quantitative factors correlated with CH_4 emission. Besides OM, starch, and NDF concentrations, also CP was significantly (negatively) correlated, but the effect was rather indirect due to concomitant changes in the percentage of other nutrients into the diet, like starch and NDF. Thus, there might be a trade-off between reducing N loss in the environment (decreasing dietary CP) and decreasing CH_4 production (higher concentrations of carbohydrates). Also, quantitative factors related to lactation performance can be highly correlated with CH_4 production. For example, milk fat concentration and enteric CH_4 are linked to rumen production of acetate. Other factors, instead, might be more correlated with CH_4 per unit of DMI or per unit of milk yield because they reflect the productive performances: for example, milk protein concentration is more correlated with milk yield and thus CH_4 intensity.

The importance of quantifying CH_4 was also stressed in chapter 5, for decision-making purposes, evaluation of mitigation strategies, and of the trend of GHG emissions over time. The dataset built for this study comprehended individual observations of lactating Holstein cows fed diets characterized by the forage crops available to farmers in the Po plain, a Mediterranean region. These diets were poorly represented in the dataset used by IPCC to build its prediction models. The diet forage basis was an important determinant of Y_m in the dataset: higher CH_4 energy loss for grass-alfalfa silage, wheat silage, and hay diets were found compared to most of the corn silage diets of the dataset. Furthermore, as underlined by the same IPCC, due to significant variation, energy digestibility values should be obtained from local scientific data wherever possible. The present study showed the lowest energy digestibility value for hay-based diets, poorly investigated by the scientific literature about enteric CH_4 from dairy cows. The evaluation of the models considered showed that an improvement for Mediterranean diets had been achieved with IPCC (2019) Tier 2 compared to the 2006 version: IPCC (2006) Tier 2 resulted in an over-prediction of CH_4 emission. The IPCC (2019) Tier 2 model, considering the average value of the whole dataset for Y_m (5.7%) and DE (70%), had best performance among the ones tested. Including data measured in vivo in Italy did not significantly improve the performance of the predicting model but just the accuracy. Nevertheless, the national emissions inventory reports have become the main instrument for reporting emissions, thus is vital to propose the best country-specific Y_m and DE values, in terms of accuracy and precision, to implement IPCC Tier 2 for the prediction of CH_4 emission.

Forages can also be exploited as high protein sources, thus reducing indirect GHG emissions from dairy cows linked to LUC. Soybean silage investigated in chapter 6 proved to be a good source of CP and EE and sustain high production in dairy cows. DMI was not affected by the treatment as well. This important variable was not measured gravimetrically in this study, but the purine derivative method gave reasonable results compared with DMI obtained through the NRC equation. This could represent an alternative and reliable method to estimate DMI. Despite the favourable results, some weak points emerged from this study. These drawbacks have to be improved in order achieve a better productivity and efficiency with the inclusion of soybean silage in the diet. In particular, the rumen protein degradability is high for legume silages, so enough readily fermentable carbohydrates have to be included in the diet to allow the rumen microbial population to use more efficiently the dietary N. In this study, probably, fermentable carbohydrates in the soybean silage diet were not sufficient, as confirmed by the lower milk protein concentration, higher milk urea, and higher N (%) excreted with urines. Another weak point was diet digestibility. Poor NDFD was probably the main driver in reducing the overall DM and OM digestibility of the soybean silage diet compared to control. There are some options to improve soybean silage quality, like using lower size varieties, hastening the harvest of the crop (but this could reduce CP digestibility), or treating chopped soybean with microbial inoculant before ensiling. Finally, a negative, even if close to zero, N retained value for soybean silage diet caused body reserve mobilization. This did not have a detrimental effect on milk production, but long-term experiments are required to investigate whether the negative N balance is confirmed and if it could negatively impact milk production.

The environmetanl sustainability of the use of sobean silage was evaluated by LCA approach. Overall, the inclusion of this silage in the diet proved to be advisable also from an environmental standpoint (chapter 7), confirming that scenarios with a diet based on home-grown forages could lower the GWP of milk production. For all the forages, the main contribution to GWP was cultivation phases (especially for GHG emissions into the air), followed by processing and transport. In this context, a forage crop with low fertilization requirements, like soybean, is advisable to enhance the sustainability of the farming system. The results regarding FEFAC compliant soybean meal (responsible soy) showed that great environmental mitigation could be achieved if soybean was not connected with illegal deforestation. It was shown that there is a linear correspondence between increasing daily diet GWP and increasing the amount of conventional soybean meal in the ration mainly due to LUC. Also, other dietary ingredients contributed to the overall GWP of daily diets or of milk production. For example, corn (forages and concentrates) contributed to emissions, so it is important to reduce the impact of other feedstuffs, not only protein sources, working on more sustainable management strategies. In conclusion, it was confirmed that dietary formulation was an interesting way to reduce the GWP of diets for dairy cows and milk, obtaining a similar production level.

9. References

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