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# Association Between Hyperketonemia and Gas Emissions Measured Using the GreenFeed System in Early-Lactation Dairy Cows

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## Simple Summary

Methane produced by dairy cows contributes substantially to greenhouse gas emissions from livestock production. Hyperketonemia is a common metabolic alteration during early lactation and may be associated with changes in enteric gas emissions. In this study, cows experiencing hyperketonemia showed higher methane emissions than cows that remained below the selected blood β-hydroxybutyrate threshold, while carbon dioxide and hydrogen emissions also differed between metabolic groups. These findings indicate an association between metabolic status and enteric gas emission patterns in early-lactation dairy cows. However, the present observational study does not establish a causal relationship or quantify the overall environmental footprint of milk production. Further research is needed to clarify the biological mechanisms underlying these associations and to determine their relevance for dairy production sustainability.

## Abstract

Hyperketonemia is a common metabolic alteration in early-lactation dairy cows, but its association with enteric gas emissions remains poorly understood. This study investigated methane ($CH_4$), carbon dioxide ($CO_2$), and hydrogen ($H_2$) emissions in relation to hyperketonemia using complementary analytical approaches. Sixty Holstein–Friesian cows underwent blood β-hydroxybutyrate (BHB) assessment at approximately 7, 14, 21, and 28 days in milk (DIM), while gas emissions were monitored using GreenFeed from 3 to 28 DIM. Cows were classified as controls (CTR; $n = 43$) or hyperketonemic (HYK; $n = 17$) based on whether BHB reached ≥1.0 mmol/L at least once. Longitudinally, HYK cows exhibited higher $CH_4$ production (+163.8 g/day), despite lower dry matter intake, alongside reduced $CO_2$ (−1182 g/day) and $H_2$ (−1.02 g/day) emissions. Higher $CH_4$ intensity and $CH_4$ yield were also observed in HYK cows. Temporal analyses revealed variations in $CH_4$ emissions surrounding the first detected hyperketonemic event. Overall, these findings highlight an association between metabolic phenotype and enteric gas emissions during early lactation. Further studies are needed to clarify underlying mechanisms and establish generalizability.

**Keywords:** ketosis; dairy cows; GHG; methane; BHB

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## 1. Introduction

Greenhouse gases (GHGs) are the main drivers of global warming, capturing heat radiated from Earth into the atmosphere [1]. The main GHGs include carbon dioxide ($CO_2$), methane ($CH_4$), nitrous oxide ($N_2O$), and fluorinated gases [2]. According to the annual reports on emissions of the Environmental Protection Agency (EPA), non-$CO_2$ gases increased by 29% between 1990 and 2015 in the United States, with $CH_4$ emissions increasing by 19% over the same period. In particular, agriculture accounted 48% of non-$CO_2$ emissions, of which 25% was directly related to livestock ($CH_4$ constituted 80%). Moreover, an increase of 10% has been estimated in $CH_4$ emissions from livestock, expected from 2015 to 2030 [3]. Furthermore, $CH_4$ is significantly more potent than $CO_2$, with a global warming potential over a 100-year time horizon ($GWP_{100}$) approximately 27.2 times that of $CO_2$ [4]. For this reason, $CH_4$ represents a critical target for strategies aimed at reducing GHG emissions and mitigating climate change.

Dairy cows play a crucial role in $CH_4$ production due to the activity of methanogenic archaea during ruminal microbial fermentations, leading to its release mainly during eructation [5,6]. Methanogenic archaea are anaerobic microorganisms that can use $CO_2$, fumarate, methanol, methylamines, ethanol, 2-butanol, and acetate to produce $CH_4$ [7,8]. Recent studies have suggested a potential link between metabolic alterations and methane-related pathways [9,10]. Moreover, metabolic disorders may indirectly influence the environmental efficiency of milk production through changes in feed intake, productive performance, and feed efficiency [11]. However, the relationship between metabolic status and directly measured individual enteric gas emissions remains poorly characterized.

The onset of lactation is a critical time for all ruminants with approximately 75% of diseases typically occurring during this period [12]. Following calving, cows frequently experience negative energy balance and systemic inflammation that predisposes them to increased mobilization of body reserves and elevated circulating ketone bodies [13,14].

Blood β-hydroxybutyrate (BHB) is commonly used as an indicator of metabolic adaptation and to identify hyperketonemia during early lactation [15]. Different BHB thresholds have been proposed depending on the purpose and population investigated. Hyperketonemia has been associated with impaired animal health, reproductive performance, and productive efficiency [16,17]. Moreover, a substantial proportion of dairy cows may experience increased BHB concentrations during early lactation (up to 56% of the herd) [18]. Beyond these direct impacts, impaired metabolic status may also influence the environmental sustainability of dairy production [19]. Differences in metabolic status may therefore also be associated with variation in enteric gas emissions and emission intensity [20].

Life cycle assessment (LCA) has become a key methodological tool to evaluate the environmental footprint of livestock production [20]. This approach quantifies the overall impact of a product by accounting for all stages of its production chain, from resource extraction and feed cultivation to farm-level emissions [21]. Despite the extensive use of LCA in dairy research, the potential contribution of metabolic disorders to the environmental efficiency of dairy production has received little attention [22,23]. However, system-level environmental assessments are conceptually distinct from direct measurements of enteric gases at the individual-animal level. To the best of our knowledge, limited information is available on the association between hyperketonemia and directly measured individual enteric gas emissions during early lactation. Characterizing this relationship may contribute to a better understanding of the links between metabolic health and enteric $CH_4$ production, while recognizing that enteric gas measurements alone do not represent the overall environmental footprint of milk production.

Regarding enteric gas emissions, several methods exist for measuring $CH_4$ emissions in ruminants, each with specific advantages and limitations. The most common techniques

are respiration chambers, the sulphur–hexafluoride ($SF_6$) tracer method, and automated head-chamber systems such as GreenFeed [24,25]. Respiration chambers provide highly precise data but are costly, labor-intensive, and may alter animal behavior. In contrast, $SF_6$ and GreenFeed allow measurements under more natural, open-air conditions and can be used on larger groups of animals, though they do not account for hindgut emissions [25,26]. Furthermore, the GreenFeed system has been validated against respiration chambers, showing deviations $\leq 3\%$ for $CH_4$ and $CO_2$ under controlled conditions [27]. Among the available techniques for measuring enteric $CH_4$, the GreenFeed system was selected for this study because it enables automated and repeated measurements in a practical on-farm setting without restraining the animals.

The hypothesis of this study was that metabolic status during early lactation would be associated with differences in enteric gas emission patterns, particularly $CH_4$ production. The aim of this study was to investigate the association between hyperketonemia and individual enteric $CH_4$, $CO_2$, and $H_2$ emissions in early-lactation dairy cows. In addition, productive performance, emission intensity and yield were evaluated to characterize the relationship between metabolic status, production, and enteric gas emissions.

## 2. Materials and Methods

### *2.1. Animals and the Study Design*

Animal care and procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and Directive 2010/63/EU for animal experiments (National law: D.L. 26/2014). The Ethics Statement was approved by the Animal Care and Use Committee of the University of Padua (protocol n. 103549/2024).

Dairy cows were enrolled from a single high-yielding Holstein–Friesian dairy herd in Piacenza province (Italy) between June and November 2024. All cows were housed in a free-stall barn with water and feed ad libitum. A total mixed ration (TMR) was provided using the Roughage Intake Control (RIC) system (Hokofarm Group, Emmeloord, The Netherlands), which allowed individual recording of daily feed intake. Daily dry matter intake (DMI; kg/day) was calculated from the TMR intake recorded by the RIC system and the dry matter content of the ration. The dry matter intake from concentrate delivered through the GreenFeed system was then added to the DMI calculated from the RIC recordings to obtain total daily DMI. Therefore, the total daily DMI values used in all statistical analyses accounted for both TMR and GreenFeed bait consumption. The composition of the TMR during the dry period and lactation is summarized in Tables 1 and 2, respectively. Diets were formulated on the basis of nutritional requirements of dairy cows according to production stage. All cows underwent a standard dry period management protocol of 60 days. After calving, milking was performed twice a day. Enrolled cows had an average parity of 2.51 ± 1.39 and the farm had an average milk production of 36 kg/cow per day.

Only dairy cows that were (*i*) multiparous, (*ii*) not affected by other diseases (ruminal acidosis, abomasal displacement, retained placenta, metritis, mastitis), and (*iii*) not under antimicrobial treatment were enrolled in the study ($n = 60$). Health status was evaluated by the veterinarian of the farm through routine clinical examination and assessment of farm health records during the transition period before enrollment. Clinical evaluation included general physical examination and routine assessment of postpartum health status to exclude animals presenting signs of metabolic or infectious diseases. Animals developing concurrent clinical diseases after enrollment were excluded from the study to minimize potential confounding effects of concurrent disease. Cows in which increased BHB concentrations were detected during the scheduled study sampling did not receive a specific treatment solely on the basis of the BHB result. The study measurements were performed as part of the experimental monitoring protocol, and mild elevations in BHB in the absence of

clinical signs did not automatically trigger therapeutic intervention according to the farm management protocol. Cows remained under routine clinical surveillance throughout the study period, and any animal developing clinical disease requiring treatment was managed according to the farm veterinary protocol.

**Table 1.** Composition of the diet for dry cows. Ingredients and composition are reported on an as-fed basis and as dry matter (DM). The chemical composition includes dry matter (DM), crude protein, crude fiber, fat forage-to-concentrate ratio (F/C), rumen undegradable protein (RUP), soluble protein, non-fibre carbohydrates (NFCs), starch, starch plus sugars, protein digested in the intestine limited by energy (PDIE) or nitrogen (PDIN), calcium (Ca), phosphorus (P), Unités Fourragères Lait (UFL) and net energy (Mcal/kg).

| Diet for Dry Cows | | |
|---|---|---|
| **Feed Ingredients** | **DM (kg)** | **DM (%)** |
| Dehulled sunflower meal | 0.93 | 6.97 |
| Soybean meal | 1.35 | 10.12 |
| Ground corn grain | 0.57 | 4.27 |
| Barley grain | 0.17 | 1.27 |
| Vitamin–mineral premix [1] | 0.07 | 0.52 |
| Wheat bran | 1.02 | 7.55 |
| Corn silage | 2.04 | 15.29 |
| Wheat straw | 7.20 | 54.01 |
| Total | 13.33 | 100.00 |
| **Parameters** | **DM (%)** | |
| DM [2] | 56.61 | |
| Crude protein | 12.59 | |
| Crude fiber | 29.31 | |
| Fat | 2.65 | |
| F/C | 64.18 | |
| RUP | 3.74 | |
| Soluble protein | 3.39 | |
| NFC | 17.65 | |
| Starch | 10.56 | |
| Starch + sugar | 12.63 | |
| PDIE | 7.92 | |
| PDIN | 8.30 | |
| Ca | 0.27 | |
| P | 0.34 | |
| | **DM** | |
| UFL (Unit/kg) | 0.72 | |
| NE (MJ/kg) | 5.39 | |

[1] Composition (DM basis): 11.5% Ca, 8.6% P, 7.5% Mg, 2.8% Na; trace elements: cupric sulfate pentahydrate (2000 mg/kg), manganous oxide (1450 mg/kg), zinc sulfate monohydrate (11,000 mg/kg), potassium iodide (95 mg/kg), sodium selenite (40 mg/kg); vitamins: vitamin A (1,500,000 IU/kg), vitamin $D_3$ (150,000 IU/kg), vitamin E (7000 mg/kg). [2] Dry matter is expressed as % of the as-fed diet; all other compositional parameters are expressed on a DM basis. The TMR included 5.00 kg/cow/day of added water on an as-fed basis.

Cow's body weight (BW) was recorded twice daily upon exiting the milking parlor using an AfiFarm 3.04E scale system (Afimilk Ltd., Kibbutz Afikim, Israel) and averaged by day. Additionally, BW change for each cow was calculated as the difference between BW at the end and at the beginning of the trial. For each animal, parity, age, daily milk yield (MY; kg/day), and daily DMI were recorded. Milk production and composition (fat, protein, lactose, %) were recorded at each milking by in-line measurement (Afilab, Afimilk, Israel). Moreover, energy-corrected milk (ECM) was subsequently calculated following standard

International Dairy Federation (IDF) and Sjaunja et al. (1991) [28] equations, respectively, in order to standardize milk output in terms of energy content and account for differences in milk composition among cows.

**Table 2.** titleComposition of the diet for lactating cows. Ingredients and composition are reported on an as-fed basis and as dry matter (DM). The chemical composition includes dry matter (DM), crude protein, crude fiber, fat forage-to-concentrate ratio (F/C), rumen undegradable protein (RUP), soluble protein, non-fibre carbohydrates (NFCs), starch, starch plus sugars, protein digested in the intestine limited by energy (PDIE) or nitrogen (PDIN), calcium (Ca), phosphorus (P), Unités Fourragères Lait (UFL) and net energy (Mcal/kg).

| **Diet for Lactating Cows** | | |
|---|---|---|
| **Feed Ingredients** | **DM (kg)** | **DM (%)** |
| Water | 0.00 | 0.00 |
| Dehulled sunflower meal | 0.93 | 3.74 |
| Soybean meal | 2.43 | 9.78 |
| Ground corn grain | 3.70 | 14.89 |
| Barley grain | 1.58 | 6.36 |
| Vitamin–mineral premix [1] | 0.45 | 1.81 |
| Hydrogenated vegetable fat | 0.20 | 0.80 |
| Sugar beet molasses | 0.70 | 2.81 |
| Wheat bran | 3.08 | 12.40 |
| Alfalfa hay | 4.08 | 16.42 |
| Corn silage | 6.80 | 27.37 |
| Wheat straw | 0.90 | 3.62 |
| Total | 24.84 | 100.00 |
| **Parameters** | **DM (%)** | |
| DM [2] | 52.42 | |
| UFL | 0.94 | |
| Crude Protein | 16.07 | |
| Crude fiber | 14.56 | |
| NDF | 33.66 | |
| Fat | 3.81 | |
| F/C | 39.90 | |
| RUP | 4.96 | |
| Soluble Protein | 4.97 | |
| NFC | 39.19 | |
| Starch | 25.93 | |
| Starch + sugars | 30.41 | |
| PDIE | 9.87 | |
| PDIN | 10.71 | |
| Ca | 0.93 | |
| P | 0.53 | |
| | **DM** | |
| UFL (Unit/kg) | 0.94 | |
| NE (MJ/kg) | 6.52 | |

[1] Composition (DM basis): 11.8% Ca, 10.8% Na, 6.5% P, 4.5% Mg; oligoelements: cupric sulfate pentahydrate (1100 mg), manganous oxide (1300 mg), zinc sulfate monohydrate (9400 mg), potassium iodide (65 mg), sodium selenite (30 mg), DL methionine (18,000 mg), microencapsulated choline chloride (20,000 mg); vitamins: vitamin A (900,000 IU/kg), vitamin D3 (150,000 IU/kg), vitamin E (3000 mg/kg). [2] Dry matter is expressed as % of the as-fed diet; all other compositional parameters are expressed on a DM basis. The TMR included 7.00 kg/cow/day of added water on an as-fed basis.

The ECM was calculated as:

$$\text{ECM} = \frac{\text{MY (kg)} \cdot [0.383 \cdot \text{fat}(\%) + 0.242 \cdot \text{protein}(\%) + 0.7832]}{3.14}$$

In addition, feed efficiency and the ratio between DMI and MY (DMI/MY, kg/kg) were then calculated.

$$\text{Feed efficiency} = \frac{\text{daily MY} \left(\frac{\text{kg}}{\text{cow}} \text{per day}\right)}{\text{total DMI} \left(\frac{\text{kg}}{\text{cow}} \text{per day}\right)}$$

A longitudinal study design was applied in this trial. All animals were evaluated at four time points (7, 14, 21, and 28 ± 2 days in milk, DIM) with clinical examinations and blood sampling. The sampling points were selected to cover the period of greatest metabolic adaptation during early lactation and to allow repeated assessment of BHB concentrations throughout the first month after calving [29]. Because blood sampling was performed at discrete intervals, transient episodes of hyperketonemia occurring between scheduled sampling points could not be excluded. Moreover, individual enteric $CH_4$, $CO_2$, and $H_2$ emissions were measured daily from 3 DIM to 28 DIM.

### *2.2. Blood Sampling and Metabolic Classification*

All blood samples were collected approximately 4 h after the morning feed delivery for each sampling time point (7, 14, 21, and 28 ± 2 days in milk, DIM). Blood samples were obtained from the jugular vessel using evacuated tubes and stored in Venosafe tubes (9 mL; Terumo Venosafe, Leuvel, Belgium) containing a clot activator. An initial determination of blood BHB concentration was performed immediately after sampling using a portable digital meter (Abbott Precision Xtra meter, Oxon, UK) and ketone test strips to provide an immediate on-farm measurement of BHB concentration. The samples were then refrigerated at 4 °C and transported in a portable freezer (CoolFreeze CFX65 W professional, Dometic, Stockholm, Sweden; minimum temperature −22 °C) at the same constant temperature to the laboratory of the Department of Animal Medicine, Production and Health (MAPS) of the University of Padua within 3 h. Thereafter, tubes were centrifuged (Heraeus Labofouge 400, Thermo Scientific, Milan, Italy) at 3.000 rpm (1.750 g-force) for 10 min at room temperature (around 20 °C). The obtained sera for each cow were equally divided into two 1.5 mL Eppendorf tubes and stored at −20 °C until biochemical analysis. Biochemical analysis was conducted using an automatic clinical chemistry analyzer (BT3500 Biotecnica Instruments, Rome, Italy) to test serum BHB concentration by the RANBUT RX Monza test (Randox, Crumlin, UK) and Non-Esterified Fatty Acids (NEFAs) by NEFA RX Monza test (Randox, Crumlin, UK).

For the primary longitudinal phenotype analysis, cows were classified according to their BHB profile across the four sampling points. Cows with BHB concentrations < 1.0 mmol/L at all scheduled measurements were classified as controls (CTR; $n = 43$) whereas cows with BHB ≥ 1.0 mmol/L at least one scheduled sampling point were classified as having experienced hyperketonemia during early lactation (HYK; $n = 17$). The HYK classification was defined retrospectively at the cow level, based on the occurrence of at least one BHB measurement ≥ 1.0 mmol/L during the observation period. Therefore, cows classified as HYK were not necessarily hyperketonemic throughout the entire study period, and gas emission measurements obtained before and after the first detection of elevated BHB were included in the primary longitudinal analysis. This classification was intended to identify cows according to their longitudinal metabolic phenotype during the first month of lactation.

The threshold of 1.0 mmol/L was selected to increase sensitivity for the identification of early or mild hyperketonemia during the transition period, as even moderate increases in BHB concentration may be associated with impaired health and productive performance [15]. Because higher thresholds, particularly BHB ≥ 1.2 mmol/L, are also commonly used to define hyperketonemia in dairy cows, an additional sensitivity analysis was performed using 1.2 mmol/L as the classification threshold.

In addition to the longitudinal phenotype classification, metabolic status was evaluated as a time-varying exposure at each scheduled BHB sampling occasion. For this analysis, each sampling occasion was classified independently according to the on-farm BHB concentration measured in the individual cow at that time point (<1.0 or ≥1.0 mmol/L). Thus, the same cow could contribute observations to different metabolic-status categories at different sampling occasions. Enteric gas measurements corresponding to these sampling occasions were then used to evaluate the association between current BHB status and gas emissions independently of the longitudinal CTR/HYK classification.

### *2.3. Enteric Gas Measurement Through GreenFeed System*

The enteric emissions (g/day) of $CH_4$, $CO_2$ and $H_2$ were measured on an individual-cow basis using one GreenFeed unit (C-Lock Inc., Rapid City, SD, USA). Cows were enrolled progressively as they calved over the study period; thus, animals were not monitored simultaneously, but were included in the GreenFeed monitoring system on a rolling basis according to their individual calving dates and days in milk. The GreenFeed unit was positioned in an alley directly adjacent to the housing area, allowing voluntary and controlled access of cows throughout the trial. The GreenFeed system measures gas concentrations in the breath and eructation of animals while they consume small portions of pelletized bait feed, ensuring an appropriate and stable head position for accurate gas sampling. Emission data were obtained usingthe GreenFeed online data management system (C-Lock Inc., Rapid City, SD, USA; https://ext.c-lockinc.com/), which automatically calculates gas fluxes after correction for airflow and background gas concentrations according to manufacturer procedures. Background air measurements were automatically recorded at regular intervals between animal visits. Before calving, cows underwent a 3-week training phase to ensure adequate habituation and visitation frequency (>1 visit/day) as recommended by C-Lock Inc. (https://docs.c-lockinc.com/GreenFeed%20Animal%20Training%20SOP.pdf (accessed on 25 September 2026)). During the training phase, the minimum interval between visits was set at 2 h; once cows were adapted and visited the units independently, the interval was increased to 3 h during the experimental phase to avoid excessive concentrate intake and to adequately capture diurnal variation in emissions. Each visit lasted approximately 3–6 min. During each visit, cows received pelleted bait feed as an attractant (ME = 2.78 Mcal/kg; CP = 184 g/kg DM; starch = 316 g/kg DM; NDF = 259 g/kg DM). The bait feed was automatically dispensed with a maximum of 8 drops per visit (35.76 ± 1.82 g/drop), consistent with procedures previously described by Moschini et al. (2025) [30]. The amount of bait consumed through the GreenFeed system was individually recorded. The corresponding dry matter intake from the concentrate delivered through GreenFeed was included in the total daily DMI calculated from the RIC recordings. Thus, total daily DMI accounted for both TMR and GreenFeed bait consumption. Overall, average bait intake was 0.81 ± 0.47 kg/d for CTR and 0.80 ± 0.47 kg/d for HYK ($p = 0.890$), indicating a comparable contribution of the GreenFeed bait feed to total feed intake between metabolic groups.

Cows were identified using RFID ear tags, allowing automatic recording of visit timing, duration, and bait feed allocation. Visits were classified according to Hristov et al. (2015) [31]. Individual GreenFeed visits were subjected to quality-control procedures before inclusion in the emission dataset. Valid visits were defined according to

the GreenFeed system criteria for head positioning threshold (HP threshold: 400–800), minimum sampling duration, and stable airflow during gas acquisition, following manufacturer operating procedures and previous methodological recommendations. The GreenFeed unit was calibrated weekly using certified calibration gases, and monthly $CO_2$ recovery tests were conducted according to manufacturer recommendations (https://docs.c-lockinc.com/Manual%20-%20GreenFeed%20-%20with%20App.pdf (accessed on 25 September 2026)).

Because GreenFeed provides spot measurements obtained during voluntary visits, the estimation of daily gas emissions depends on both the frequency and temporal distribution of valid measurements. Previous studies have reported average visitation frequencies ranging from 1.2 to 4.6 visits/day, highlighting the importance of adequate diurnal coverage for reliable estimation of daily enteric gas emissions under practical production conditions [25]. Considering the variability in visitation frequency reported in the literature, cow-days with at least one valid GreenFeed visit were included in the primary analysis. To assess the robustness of the findings to a more restrictive visitation criterion, an additional sensitivity analysis was designed by restricting the dataset to cow-days with at least two valid GreenFeed visits. The distribution of GreenFeed visits across the 24 h period was also examined to assess diurnal coverage of the measurements. This approach has been widely adopted in field-based studies and allows non-invasive and repeatable quantification of enteric GHG under practical production conditions [32–34]. Previous studies reported average visitation frequencies ranging from 1.2 to 4.6 visits/d and demonstrated that measurements distributed across the diurnal cycle can provide reliable estimates of daily enteric gas emissions under practical production conditions [25].

The $CH_4$ emissions measured by the GreenFeed unit were converted $CH_4$-derived $CO_2$-equivalents ($CO_2e$). Conversion was based on the 100-year global warming potential ($GWP_{100}$) of $CH_4$ as reported by the Intergovernmental Panel on Climate Change (IPCC). The following equation was applied:

$$CO_2e\ (kg) = CH_4\ (kg) \times GWP_{CH4}$$

According to the IPCC Sixth Assessment Report (IPCC, 2021) [35], a $GWP_{100}$ value of 27.2 was used. These values represent $CO_2$-equivalents derived from enteric $CH_4$ only and should not be interpreted as the overall carbon footprint of the animals or milk production system. The $CH_4$ intensity ($CH_4$/MY, g/kg milk), $CO_2$ intensity ($CO_2$/MY, g/kg milk), and H2 intensity ($H_2$/MY, g/kg milk) were calculated based on daily MY. $CH_4$ yield was calculated as the ratio between $CH_4$ emissions and DMI ($CH_4$/DMI, g/kg DM).

### *2.4. Statistical Analysis*

The minimum sample size was estimated a priori using G*Power software (version 3.1.9.7) (http://www.gpower.hhu.de/ (accessed on 25 September 2026)). The calculation was based on previously reported differences in daily $CH_4$ production between dairy cows characterized by divergent methane-emission phenotypes and monitored using the GreenFeed system by Denninger et al. 2019 [36]. In that study, mean $CH_4$ production was 426 ± 34.3 g/day in low-emitting cows and 478 ± 39.4 g/day in high-emitting cows. Based on these values, the standardized effect size (Cohen's d) was calculated as 1.41. Assuming a two-sided significance level of $\alpha = 0.05$, a statistical power of 0.95, and equal allocation between two independent groups, the estimated minimum sample size was 30 cows (15 cows per group).

To assess potential temporal imbalances between metabolic groups, the distribution of cows across months of enrolment was compared between the CTR and HYK groups using Pearson's chi-square test of independence. This analysis was performed to evaluate

whether the two groups were similarly represented across the study period and to identify potential differences in enrolment timing that could contribute to seasonal confounding.

All statistical analyses were performed using R software ("rcmdr" package, version 4.5.1) [37]. The normal distribution and homogeneity of variance were confirmed by Shapiro–Wilk and Levene tests. Model assumptions were evaluated by inspecting standardized residuals, Q–Q plots, and residuals vs. fitted values plots. Normality and homoscedasticity were assessed through visual examination of Q–Q plots and residual patterns, respectively. Outliers were screened using standardized residual diagnostics and residual plots; each case was individually examined. No influential or aberrant observations were detected, and no data were excluded from the analyses. Longitudinal data were analysed using linear mixed-effects models. Statistical significance was set at $p \leq 0.05$. Results with $0.05 < p < 0.10$ were reported as statistical trends.

The overall study design, including the experimental timeline, data collection procedures, and statistical analyses, is schematically illustrated in Figure 1.

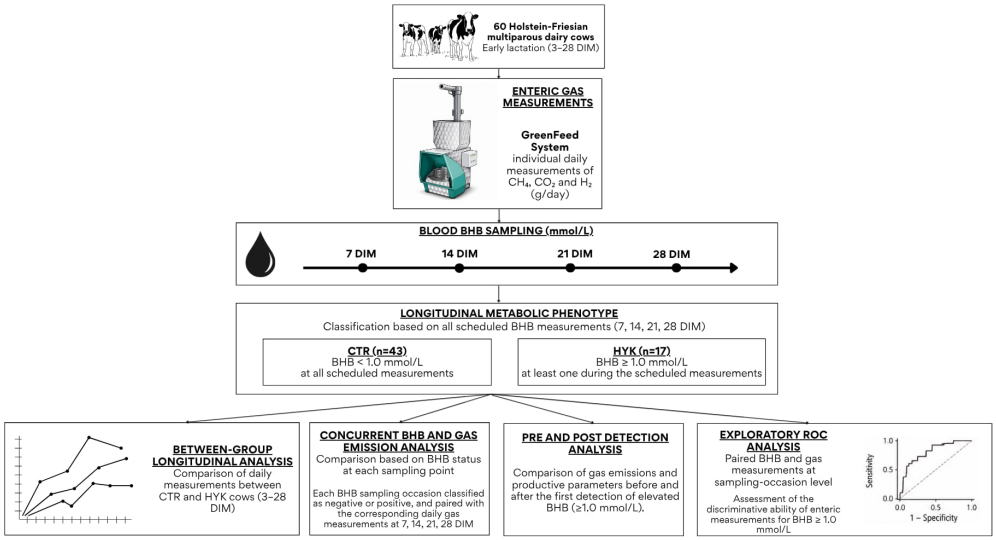



**Figure 1.** Schematic overview of the study design and statistical analyses.

#### 2.4.1. Longitudinal Metabolic Phenotype

Cows were classified into two metabolic groups according to their blood BHB concentrations measured at 7, 14, 21, and 28 DIM. Cows with BHB concentrations < 1.0 mmol/L at all scheduled sampling occasions were classified as controls (CTR), whereas cows with at least one BHB concentration ≥ 1.0 mmol/L were classified as hyperketonemic (HYK). This classification was maintained throughout the longitudinal analysis from 3 to 28 DIM.

For absolute $CH_4$, $CO_2$, and $H_2$ emissions (g/day), the primary longitudinal analyses were performed using linear mixed-effects models including metabolic phenotype (CTR vs. HYK), DIM, their interaction, and MY as fixed effects, with cows included as a random intercept. MY was included to account for differences in milk production and to evaluate the association between metabolic phenotype and absolute gas emissions conditional on productive performance. To assess the robustness of these findings, corresponding models without MY adjustment were fitted as sensitivity analyses using the same observations. For $CH_4$, $CO_2$, and $H_2$ emission intensities (g/kg milk), MY was not included as an additional covariate because milk production was already incorporated into the denominator of the respective response variables. Similarly, $CH_4$ yield (g/kg DMI) was analyzed without MY adjustment, as the objective was to evaluate methane emissions relative to DMI. The same unadjusted model structure was applied to body weight (BW), BW change, MY, ECM, DMI,

feed efficiency, DMI-to-MY ratio, and milk fat, protein, and lactose concentrations from 3 to 28 DIM. In contrast, the longitudinal patterns of BHB and NEFA concentrations were evaluated only at the four time-points (7, 14, 21, and 28 DIM).

The linear mixed model used to evaluate longitudinal changes in the investigated variables according to metabolic phenotype was:

$$y_{ij} = \beta_0 + \beta_1\, Group_i + \beta_2\, Time_{ij} + \beta_3\, (Group{\cdot}Time)_{ij} + \sum_{k=1}^{m} .\, \beta_k Cov_{k,ij} + u_i + \varepsilon_{ij}$$

where *yij* represents the dependent variable measured on animal *i* at time *j*. The $\beta_0$ is the overall intercept, while $\beta_1$, $\beta_2$ and $\beta_3$ represent fixed effects of group (CTR vs. HYK), time (DIM), and their interaction, respectively. Time was included as a categorical variable with 26 levels (from 3 to 28 DIM). Daily MY was included as a covariate in the primary models for absolute $CH_4$, $CO_2$, and $H_2$ emissions, whereas the corresponding sensitivity models excluded this covariate. Models evaluating emission intensities and $CH_4$ yield were also fitted without MY adjustment. The term *ui* represents the random intercept for animal *i*, accounting for repeated measurements within cows, and *εij* is the residual error term.

Furthermore, to evaluate the robustness of the findings to the selected BHB threshold, a sensitivity analysis was performed using the more restrictive threshold of ≥1.2 mmol/L for hyperketonemia. For this analysis, cows were reclassified according to their longitudinal BHB profile as remaining below 1.2 mmol/L at all scheduled sampling points or having at least one BHB concentration ≥ 1.2 mmol/L during the first month of lactation. The primary longitudinal mixed-effects models for $CH_4$, $CO_2$, and $H_2$ production were then repeated using this alternative metabolic classification, while maintaining the same fixed- and random-effects structure as in the primary analysis.

#### 2.4.2. Association Between Concurrent BHB Status and Gas Emissions

This analysis aimed to investigate the association between BHB status at individual sampling occasions and concurrent gas emissions, independently of the metabolic phenotype assigned to each cow in the longitudinal analysis.

Each BHB measurement obtained at 7, 14, 21, and 28 DIM was classified as either below (BHB < 1.0 mmol/L) or above (BHB ≥ 1.0 mmol/L) the predefined threshold. Unlike the longitudinal metabolic phenotype analysis, metabolic status was assigned separately at each sampling occasion, allowing individual cows to contribute observations to both categories. Each paired BHB–gas measurement was considered an individual observation.

The association was evaluated using separate linear mixed-effects models, including metabolic BHB status as fixed effects and cow as a random intercept to account for repeated measurements within animal. DIM and MY were treated as covariates.

#### 2.4.3. Pre- and Post-Hyperketonemia Detection Analysis

A third statistical approach was applied to investigate overall differences in emissions and production before and after the detection of hyperketonemia only on cows in the HYK group. Measurements collected from 8 days before to 8 days after diagnosis were classified into two periods: Pre-first detection (days −8 to −1) and Post-first detection (day 0 to +8). Linear mixed-effects models were fitted to compare mean values between periods for $CH_4$, $CO_2$ and $H_2$ production; MY, DMI, $CH_4$, $CO_2$ and $H_2$ intensity (on milk basis); and DMI:MY. In this model, period (Pre vs. Post) was included as fixed effect, while animal was included as a random effect.

Another model was used to better understand the trend around detection, focusing on single days. The 17 levels, from 8 days before to 8 days after diagnosis were used as fixed effect, and animal as random and repeated effects. This approach allowed for a detailed assessment of day-by-day changes in emissions and production parameters around the

diagnosis of SCK. In both cases, a post hoc pairwise comparison among LS-means was performed using Tukey correction. Because BHB concentrations were assessed only at scheduled sampling occasions, day 0 represents the first detection of hyperketonaemia and should not be interpreted as the biological onset of the metabolic alteration. Because enrolment occurred continuously throughout the study period, the distribution of CTR and HYK cows according to calendar month was examined to assess potential imbalance in enrolment timing. Differences in the monthly distribution of cows between metabolic phenotypes were evaluated using a chi-square test.

#### 2.4.4. Receiver Operating Characteristic (ROC) Curve Analysis

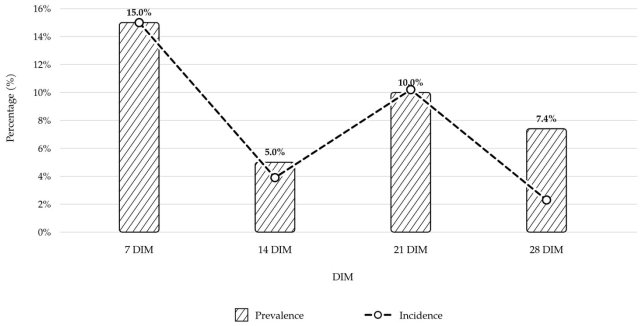
An exploratory receiver operating characteristic (ROC) analysis was performed to investigate the discriminatory ability of enteric gas variables in relation to metabolic status at each scheduled BHB sampling occasion. This analysis was performed using MedCalc statistical software (version 23.1.6) (MedCalc Software Ltd., Ostend, Belgium). The unit of analysis was the individual sampling occasion, rather than the individual cow. Each BHB measurement was paired with the corresponding $CH_4$, $CO_2$, and $H_2$ production measurements, as well as $CH_4$ intensity and yield ($CH_4$/DMI), obtained on the same sampling day. No cow-level averaging or aggregation was performed for the ROC analysis, and individual cows could contribute repeated observations across sampling occasions. The area under the ROC curve (AUC) was calculated for each variable, and 95% confidence intervals (CIs) were estimated using a cow-level cluster bootstrap procedure with 2000 replicates to account for within-cow correlation arising from repeated measurements. In each bootstrap replicate, cows were randomly sampled with replacement, retaining all available observations from each selected cow, and the AUC was recalculated. Exploratory data-derived thresholds were identified using the Youden index, and the corresponding sensitivity and specificity were calculated. The uncertainty associated with the optimal thresholds was also assessed using the cow-level cluster bootstrap procedure. Because the ROC curves and thresholds were derived and evaluated within the same study population, these thresholds were considered exploratory and were not interpreted as validated diagnostic cut-offs.

## 3. Results

The temporal distribution of enrolled cows was evaluated to assess potential differences in the two metabolic groups. Both groups were represented throughout the study period, and their distribution across calendar months did not differ significantly ($\chi^2 = 1.39$, df = 4; $p = 0.846$). Specifically, 17 CTR and 9 HYK cows were enrolled between June and August, whereas 26 CTR and 8 HYK cows were enrolled between September and October. These findings indicated no evidence of a seasonal imbalance between the two metabolic groups. The values of incidence and prevalence of hyperketonemia and the results from the linear mixed model of NEFA and BHB at 7, 14, 21 and 28 DIM are reported in Figure 2. Most new cases peaked at 7 and 21 DIM. For NEFA, a significant effect of group ($p = 0.013$) and time ($p = 0.006$) was observed. Cows in the HYK group exhibited higher NEFA concentrations compared with CTR at 7 and 14 DIM. The NEFA levels increased in both groups at 28 DIM, indicating a temporal rise during early lactation. Regarding BHB, a significant group effect was also detected ($p < 0.001$), with higher concentrations in HYK cows at 7 DIM and 21 DIM.

During the experimental period, the average GreenFeed visitation frequency was 2.83 ± 1.64 visits/day in CTR cows and 2.80 ± 1.65 visits/day in HYK cows. A total of 3392 valid visits were recorded, including 2434 in CTR cows and 958 in HYK cows. At least one valid GreenFeed measurement was available for 860 cow-days (77.0%) in CTR cows and 342 cow-days (77.4%) in HYK cows. A detailed summary of GreenFeed visitation frequency and data availability is provided in Supplementary Table S1.

Cow-days with at least one valid visit were included in the primary analysis, whereas the subset with at least two valid visits was considered for the sensitivity analysis (Supplementary Table S2). For $CH_4$ emissions, the estimated difference between HYK and CTR cows was +158.4 g/day ($p = 0.012$), compared with +163.8 g/day ($p = 0.039$) in the analysis including all available cow-days. Similarly, the estimated difference in $CO_2$ emissions was −1105 g/day ($p = 0.027$), compared with −1182 g/day ($p = 0.007$) in the primary analysis. For $H_2$ emissions, the estimated difference was −0.910 g/day ($p = 0.002$), compared with −1.020 g/day ($p = 0.009$) in the primary analysis. Overall, the between-group differences remained consistent in direction and similar in magnitude across the two analytical approaches, indicating that the observed associations between metabolic phenotype and enteric gas emissions were not substantially affected by applying the more restrictive criterion of at least two valid GreenFeed visits per cow-day.




| Parameter | Group | 7 DIM | 14 DIM | 21 DIM | 28 DIM | SEM [1] | *p*-Values | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Group | Time | Interaction (Group × Time) |
| **NEFA (mEq/L)** | CTR | 0.46 $^{y,b}$ | 0.43 $^{y,b}$ | 0.42 $^{b}$ | 0.65 $^{a}$ | 0.06 | 0.013 | 0.006 | 0.201 |
| | HYK | 0.71 $^{x,a}$ | 0.59 $^{x,ab}$ | 0.44 $^{b}$ | 0.63 $^{a}$ | 0.08 | | | |
| **BHB (mmol/L)** | CTR | 0.56 $^{y}$ | 0.51 | 0.53 $^{y}$ | 0.59 | 0.07 | <0.001 | 0.538 | 0.209 |
| | HYK | 0.91 $^{x,a}$ | 0.66 $^{b}$ | 0.77 $^{x,b}$ | 0.79 $^{ab}$ | 0.11 | | | |

[1] Standard Error of Means;
$^{a,b}$ Indicate significant changes within row; $^{x–z}$ indicate significant changes within column.
[1] Standard Error of Means;
$^{x–y}$ Indicate significant changes within column; $^{a-b}$ indicate significant changes within row.

**Figure 2.** Prevalence and incidence (%) of hyperketonemia in dairy cows during the four time-points (7, 14, 21, and 28 days in milk, DIM). Bars represent the prevalence of hyperketonemia, while the dashedline indicates the incidence of new cases within the same intervals. Below, results from linear mixed model (*p*-values) and least square means for non-esterified fatty acids (NEFAs) and BHB. Values are expressed as least-squares means (LSMeans) ± standard error of the mean (SEM).

The longitudinal analysis of daily gas emissions was performed to investigate differences between CTR and HYK cows throughout early lactation (3–28 DIM). The primary longitudinal analyses of absolute $CH_4$, $CO_2$, and $H_2$ emissions were performed using linear mixed-effects models adjusted for daily MY. To assess the robustness of the findings, corresponding models without MY adjustment were fitted as sensitivity analyses (Table 3). Adjustment for MY did not materially alter the estimated between-group differences in daily gas emissions. For $CH_4$, the estimated difference between HYK and CTR cows remained +167.7 and +163.8 g/day in the unadjusted and MY-adjusted models, respectively, although the significance of the group effect decreased from $p < 0.001$ to $p = 0.039$. Similarly, the estimated between-group differences for $CO_2$ were −1278 and −1182 g/day in the unadjusted and MY-adjusted models, respectively, while those for $H_2$ were −0.903 and −1.02 g/day. The group effect remained statistically significant for both $CO_2$ ($p = 0.007$) and $H_2$ ($p = 0.009$) after MY adjustment. MY was significantly associated with $CO_2$ ($p < 0.0001$) and $H_2$ ($p = 0.025$) emissions, but not with $CH_4$ emissions ($p = 0.985$). Overall, these

findings suggest that the estimated differences in gas emissions between metabolic groups were relatively stable following adjustment for MY, although the temporal pattern of $CO_2$ emissions differed between groups in the adjusted model. Least-squares means ± standard errors (LSMeans ± SE) of enteric gas emissions and related ratio according to group division (HYK vs. CTR) in early lactation are presented in Table 4. In the primary analyses, models for absolute $CH_4$, $CO_2$, and $H_2$ production were adjusted for MY whereas models for emission intensities and $CH_4$ yield were not adjusted for MY. The results of the longitudinal analyses of BW, BW change, MY, ECM, DMI, feed efficiency, DMI-to-MY ratio (kg/kg), and milk fat, protein, and lactose concentrations are presented in Table 5.

**Table 3.** Estimated between-group differences and 95% Confidence Interval (95% CI) are expressed as HYK − CTR contrast, with positive values indicating higher emissions in hyperketonemic cows and negative values indicating lower emissions. Models included phenotype group (CTR and HYK), time (days in milk, DIM), and their interaction as fixed effects. MY-adjusted models additionally included daily milk yield as a covariate. MY-adjusted models were considered the primary analyses for absolute gas emissions, whereas unadjusted models were performed as sensitivity analyses. The *p*-values reported refer to the overall tests of the corresponding fixed effects in the models.

| Gas | Model | Estimated Difference (HYK − CTR) g/day | 95% CI (g/day) | Group | Time | Interaction | Milk |
|---|---|---|---|---|---|---|---|
| $CH_4$ | Primary (MY-adjusted) | +163.8 | 130; 197.5 | 0.039 | <0.001 | 0.195 | 0.985 |
| $CH_4$ | Sensitivity (Unadjusted) | +167.7 | 135.4; 200 | <0.001 | <0.001 | 0.103 | — |
| $CO_2$ | Primary (MY-adjusted) | −1182 | −2157; −207 | 0.007 | <0.001 | 0.006 | <0.0001 |
| $CO_2$ | Sensitivity (Unadjusted) | −1278 | −2195; −361 | <0.001 | <0.001 | 0.222 | — |
| $H_2$ | Primary (MY-adjusted) | −1.020 | −1.392; −0.642 | 0.009 | 0.382 | 0.517 | 0.025 |
| $H_2$ | Sensitivity (Unadjusted) | −0.903 | −1.352; −0.454 | <0.001 | 0.457 | 0.652 | — |

**Table 4.** Model-estimated least-squares means ± standard error of the mean (LSMeans ± SEM) for methane ($CH_4$), carbon dioxide ($CO_2$), dihydrogen ($H_2$), milk yield (MY), dry matter intake (DMI), methane, carbon dioxide intensity, dihydrogen intensity on milk basis, methane yield ($CH_4$/DMI) in control (CTR, $n = 43$) and with hyperketonemia (HYK, $n = 17$), from 3 to 28 days in milk (DIM). LSMeans were estimated from linear mixed-effects models and averaged across the 3–28 DIM observation period. For absolute $CH_4$, $CO_2$, and $H_2$ emissions, LSMeans and corresponding *p*-values were obtained from the primary MY-adjusted models. For emission intensities and $CH_4$ yield, estimates were obtained from models without MY adjustment.

| Parameters | CTR | HYK | *p*-Values | | | |
|---|---|---|---|---|---|---|
| | | | Group | Time | Interaction (Group × Time) | Milk |
| **$CH_4$ Production (g/day)** | 238 ± 17.5 $^{x}$ | 402 ± 25.9 $^{y}$ | 0.039 | <0.001 | 0.195 | 0.985 |
| **$CH_4$ Intensity (g/kg milk)** | 7.71 ± 0.76 $^{y}$ | 12.9 ± 1.16 $^{x}$ | <0.001 | <0.001 | <0.001 | - |
| **$CH_4$ Yield (g/kg DM)** | 13.5 ± 2.76 $^{y}$ | 25.5 ± 4.06 $^{x}$ | <0.001 | 0.781 | <0.001 | - |
| **$CO_2$ Production (g/day)** | 12,180 ± 412 $^{x}$ | 10,998 ± 587 $^{y}$ | 0.007 | <0.001 | 0.006 | <0.001 |
| **$CO_2$ Intensity (g/kg milk)** | 370 ± 28.8 $^{x}$ | 316 ± 42.8 $^{y}$ | 0.009 | <0.001 | 0.687 | - |
| **$H_2$ Production (g/day)** | 2.01 ± 0.36 $^{x}$ | 1.01 ± 0.51 $^{y}$ | 0.009 | 0.382 | 0.517 | 0.025 |
| **$H_2$ Intensity (g/kg milk)** | 0.058 ± 0.014 $^{x}$ | 0.026 ± 0.016 $^{y}$ | 0.025 | <0.001 | 0.866 | - |

$^{x,y}$ indicate significant changes between groups.

The statistical analysis revealed that several parameters were significantly influenced by the group division. The result of longitudinal analysis are shown in Figure 3. A group effect (HYK vs. CTR) was detected for $CH_4$ ($p = 0.039$), $CO_2$ ($p = 0.007$) and $H_2$ ($p = 0.009$) production, DMI ($p = 0.002$), $CH_4$ intensity ($p < 0.001$), CO2 intensity ($p = 0.009$) and $H_2$ intensity on milk basis ($p = 0.025$), DMI:MY ($p < 0.001$), and $CH_4$ yield ($p < 0.001$). Finally, a significant Group × Time interaction was evidenced for $CO_2$ ($p = 0.006$), $CH_4$ intensity on milk basis ($p < 0.001$), DMI:MY ($p = 0.035$), and $CH_4$ yield ($p < 0.001$). During the period

between 3 and 28 DIM, HYK cows showed higher $CH_4$ production, both in absolute terms and when expressed relative to MY ($CH_4$/MY) and DMI ($CH_4$/DMI). In contrast, $CO_2$ and $H_2$ emissions, as well as $CO_2$/MY and $H_2$/MY ratios, were lower in HYK cows compared with CTR. The DMI was also significantly lower in the HYK group. Milk composition (fat, protein and lactose) was not affected by metabolic status. A significant time effect was observed for most productive and compositional traits, including BW and milk composition (fat, protein, and lactose).

**Table 5.** Model-estimated least-squares means ± standard error of the mean (LSMeans ± SEM) for body weight (BW), BW change, milk yield (MY), energy-corrected milk (ECM), dry matter intake (DMI), feed efficiency, the ratio between dry matter intake and milk yield (DMI:MY), milk fat, milk protein and lactose in control (CTR, $n = 43$) and animals with hyperketonemia (HYK, $n = 17$), from 3 to 28 days in milk (DIM).

| Parameters | CTR | HYK | *p*-Values Group | Time | Interaction |
|---|---|---|---|---|---|
| **BW (kg)** | 613 ± 8.57 | 611 ± 13.5 | 0.470 | <0.001 | 0.057 |
| **BW change (kg)** | −23 ± 7.51 | −28.67 ± 8.44 | 0.206 | — | — |
| **MY (kg/day)** | 36 ± 1.78 | 35.4 ± 2.80 | 0.787 | <0.001 | 0.288 |
| **ECM (kg/day)** | 39.8 ± 2.64 | 45.03 ± 4.14 | 0.840 | <0.001 | 0.324 |
| **DMI (kg/day)** | 21.9 ± 1.16 $^{x}$ | 19.0 ± 1.72 $^{y}$ | 0.002 | <0.001 | 0.549 |
| **Feed Efficiency (kg milk/kg DM)** | 1.72 ± 0.23 | 2.14 ± 0.34 | 0.115 | 0.187 | 0.034 |
| **DMI:MY (kg/kg)** | 0.61 ± 0.05 $^{x}$ | 0.49 ± 0.08 $^{y}$ | <0.001 | <0.001 | 0.035 |
| **Milk Fat (%)** | 4.04 ± 0.15 | 4.23 ± 0.22 | 0.437 | <0.001 | 0.336 |
| **Milk Protein (%)** | 3.46 ± 0.14 | 3.29 ± 0.20 | 0.467 | 0.007 | 0.955 |
| **Lactose (%)** | 4.75 ± 0.36 | 4.74 ± 0.28 | 0.586 | 0.015 | 0.996 |

$^{x,y}$ indicate significant changes between groups.

Furthermore, to assess the robustness of the longitudinal findings, a sensitivity analysis was performed using a higher BHB threshold (≥1.2 mmol/L). Increasing the threshold resulted in the reclassification of six cows from HYK to CTR, yielding 11 HYK and 49 CTR cows. The direction of the associations remained consistent with the primary longitudinal analysis. At the ≥1.2 mmol/L threshold, HYK cows exhibited higher daily $CH_4$ emissions than CTR cows (estimated difference: +178 g/day; 95% CI: 105 to 252; $p < 0.001$), whereas $CO_2$ (−2097 g/day; 95% CI: −3290 to −903; $p < 0.001$) and $H_2$ emissions (−0.837 g/day; 95% CI: −1.650 to −0.019; $p = 0.045$) were significantly lower. These findings suggest that the direction of the associations between hyperketonemia and gas emissions was maintained when a more restrictive BHB threshold was applied.

Regarding the analysis of concurrent BHB status and gas emissions, the results are reported in Table 6. Measurement occasions with BHB concentrations ≥ 1.0 mmol/L were associated with significantly higher daily $CH_4$ emissions compared with those with BHB concentrations < 1.0 mmol/L. The estimated difference was +90.68 g/day ($p = 0.012$). In contrast, $CO_2$ and $H_2$ emissions were numerically lower when BHB concentrations were ≥1.0 mmol/L, with estimated differences of −1.124 g/day ($p = 0.070$) and −0.975 g/day ($p = 0.076$), respectively, although neither association reached statistical significance (Table 6). These results support an association between BHB status at the sampling occasion and $CH_4$ emissions, while avoiding the assumption that cows classified as HYK remained hyperketonemic throughout the monitoring period.

When comparing means before and after the first detection of hyperketonemia for the HYK group (Table 7), significant differences were observed for several variables. The $CH_4$ emissions were significantly higher in the Post-detection period compared with Pre-detection ($p < 0.001$). Similarly, $CO_2$ emissions increased after diagnosis ($p = 0.011$). The MY and DMI were also significantly greater in the Post-detection period, with increases of 2.76 ± 1.35 kg/day ($p = 0.045$) and 4.35 ± 0.91 kg/day ($p < 0.001$), respectively. When emissions were expressed relative to MY, $CO_2$ intensity decreased significantly in the Post-

detection period (−52.4 ± 26.2 g/kg; $p = 0.048$). Conversely, the $CH_4$ yield was significantly higher after detection (+13.8 ± 5.66 g/kg; $p = 0.016$).

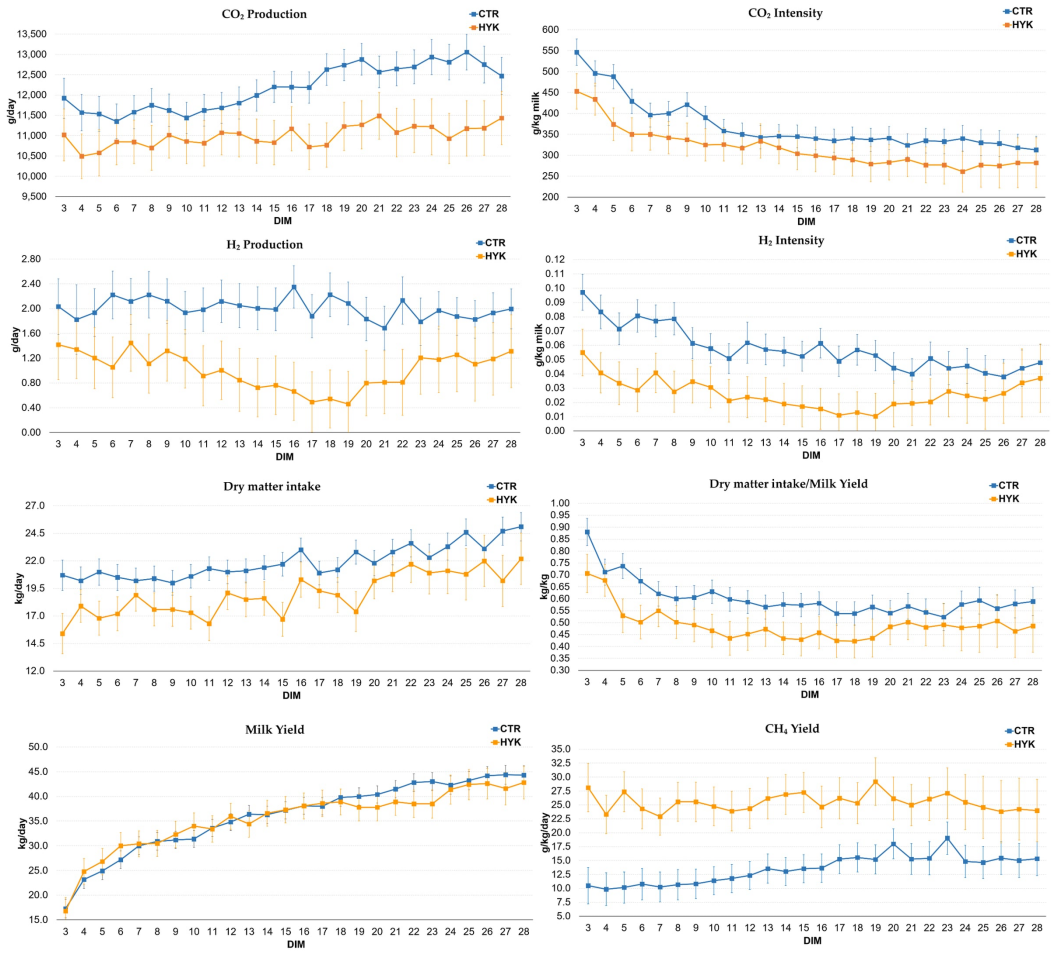



**Figure 3.** Daily production of methane ($CH_4$; g/day), carbon dioxide ($CO_2$; g/day) and dihydrogen ($H_2$; g/day), methane intensity ($CH_4$/MY, g/kg), carbon dioxide intensity ($CO_2$/MY, g/kg), dihydrogen intensity ($H_2$/MY, g/kg), daily milk yield (MY; kg/day), dry matter intake (DMI; kg/day), the ratio between dry matter intake and milk yield (DMI/MY, kg/kg), methane yield ($CH_4$/DMI, g/kg) in control (CTR, $n = 43$) and animals with hyperketonemia (HYK, $n = 17$), from 3 to 28 days in milk (DIM).

**Table 6.** Association between time-varying BHB status and enteric gas emissions in early-lactation dairy cows. Estimated differences compare observations with BHB ≥ 1.0 mmol/L against those with BHB < 1.0 mmol/L. Positive values indicate higher emissions in the hyperketonemic observations.

| Gas | Estimated Difference (g/day) | 95% CI | *p*-Value |
|---|---|---|---|
| $CH_4$ | +90.68 | 21.24; 160.12 | 0.012 |
| $CO_2$ | −1124 | −2454.80; 206.80 | 0.070 |
| $H_2$ | −0.975 | −2.045 to 0.095 | 0.076 |

The analysis included 60 cows (43 CTR and 17 HYK), contributing a total of 240 paired BHB–GreenFeed observations. Of these, 21 observations were classified as BHB-positive (BHB ≥ 1.0 mmol/L) and 219 as BHB-negative (BHB < 1.0 mmol/L). Among HYK cows, 13 contributed one hyperketonemic observation each, whereas 4 cows contributed two hyperketonemic observations each.

**Table 7.** Estimated marginal means (least-squares means, LSMeans) ± standard error (SE) of gas emissions and productive parameters in hyperketonemic cows ($n = 17$) during the pre-detection (days −8 to −1) and post-detection (days 0 to +8) periods. Estimated differences were obtained from linear mixed-effects models.

| Parameters | Pre-First Detection | After First Detection | Difference (Post–Pre) | *p*-Values |
|---|---|---|---|---|
| $CH_4$ Production (g/day) | 343 ± 17.7 | 399 ± 17.4 | 55.9 ± 9.32 | <0.001 |
| $CH_4$ Intensity (g/kg milk) | 12.5 ± 1.42 | 11.1 ± 1.31 | −1.39 ± 1.01 | 0.172 |
| $CH_4$ yield (g/kg DM) | 25.5 ± 6.14 | 39.4 ± 6.69 | 13.8 ± 5.66 | 0.016 |
| $CO_2$ Production (g/day) | 10,507 ± 486 | 11,117 ± 477 | 610 ± 237 | 0.011 |
| $CO_2$ Intensity (g/kg milk) | 358 ± 26.4 | 306 ± 22.1 | −52.4 ± 26.2 | 0.048 |
| $H_2$ Production (g/day) | 0.98 ± 0.189 | 1.12 ± 0.184 | 0.135 ± 0.11 | 0.220 |
| $H_2$ Intensity (g/kg milk) | 0.031 ± 0.005 | 0.03 ± 0.005 | −0.002 ± 0.004 | 0.653 |
| MY (kg/day) | 34.2 ± 2.66 | 37.0 ± 2.57 | 2.76 ± 1.35 | 0.045 |
| DMI (kg/day) | 14.4 ± 1.75 | 18.8 ± 1.70 | 4.35 ± 0.908 | <0.001 |
| DMI:MY (kg/kg) | 0.507 ± 0.03 | 0.482 ± 0.04 | −0.025 ± 0.04 | 0.588 |

The subsequent day-by-day analysis, covering the period from 8 days before to 8 days after the first detection of hyperketonemia, revealed a significant effect of relative day on $CH_4$ production ($p = 0.002$), MY ($p < 0.001$), and DMI ($p = 0.006$). Daily MY was significantly associated with $CO_2$ production ($p = 0.001$; estimated coefficient: +58.88 g/day per kg/day of milk) and DMI ($p = 0.028$; estimated coefficient: +0.15 kg/day per kg/day of milk). As shown in Figure 4, $CH_4$ emissions increased around the first detection of hyperketonemia, reaching their highest values at days +1 and +2 and remaining elevated for approximately five days before gradually declining. The milk daily production decreased to 28.9 kg/day at day −2, followed by a subsequent increase. The DMI showed moderate fluctuations before the first detection of hyperketonemia, followed by an increase at day 0 (19.94 kg/day) and a progressive rise thereafter, reaching its highest value at day +8. This event-centered analysis describes temporal variations in gas emissions around the first scheduled detection of hyperketonemia. However, these changes should be interpreted cautiously, as they may also reflect physiological changes associated with the progression of early lactation.

The results of the exploratory ROC analyses are presented in Figure 5. $CH_4$ production showed the highest apparent discriminatory ability for identifying sampling occasions with blood BHB concentrations ≥ 1.0 mmol/L, with an AUC of 0.882 (95% CI: 0.818–0.938). The optimal exploratory threshold identified using the Youden index was ≥336.99 g/day, corresponding to a sensitivity of 100% and a specificity of 81.9%.

$CO_2$ production showed limited discriminatory ability (AUC = 0.626; 95% CI: 0.449–0.802). The corresponding exploratory threshold was ≤12,657.82 g/day, with a sensitivity of 90.0% and a specificity of 35.5%. $H_2$ production showed moderate discriminatory ability (AUC = 0.736; 95% CI: 0.587–0.867), with an exploratory threshold of ≤0.75 g/day, corresponding to a sensitivity of 70.0% and a specificity of 73.2%.

Among $CH_4$-related variables, $CH_4$ intensity relative to milk yield ($CH_4$/MY) showed an AUC of 0.867 (95% CI: 0.774–0.947), with an exploratory threshold of ≥8.21 g/kg milk, corresponding to a sensitivity of 100% and a specificity of 70.5%. Similarly, $CH_4$ yield relative to dry matter intake ($CH_4$/DMI) showed an AUC of 0.868 (95% CI: 0.787–0.936). The corresponding exploratory threshold was ≥15.94 g/kg DMI, with a sensitivity of 90.0% and a specificity of 78.0%.

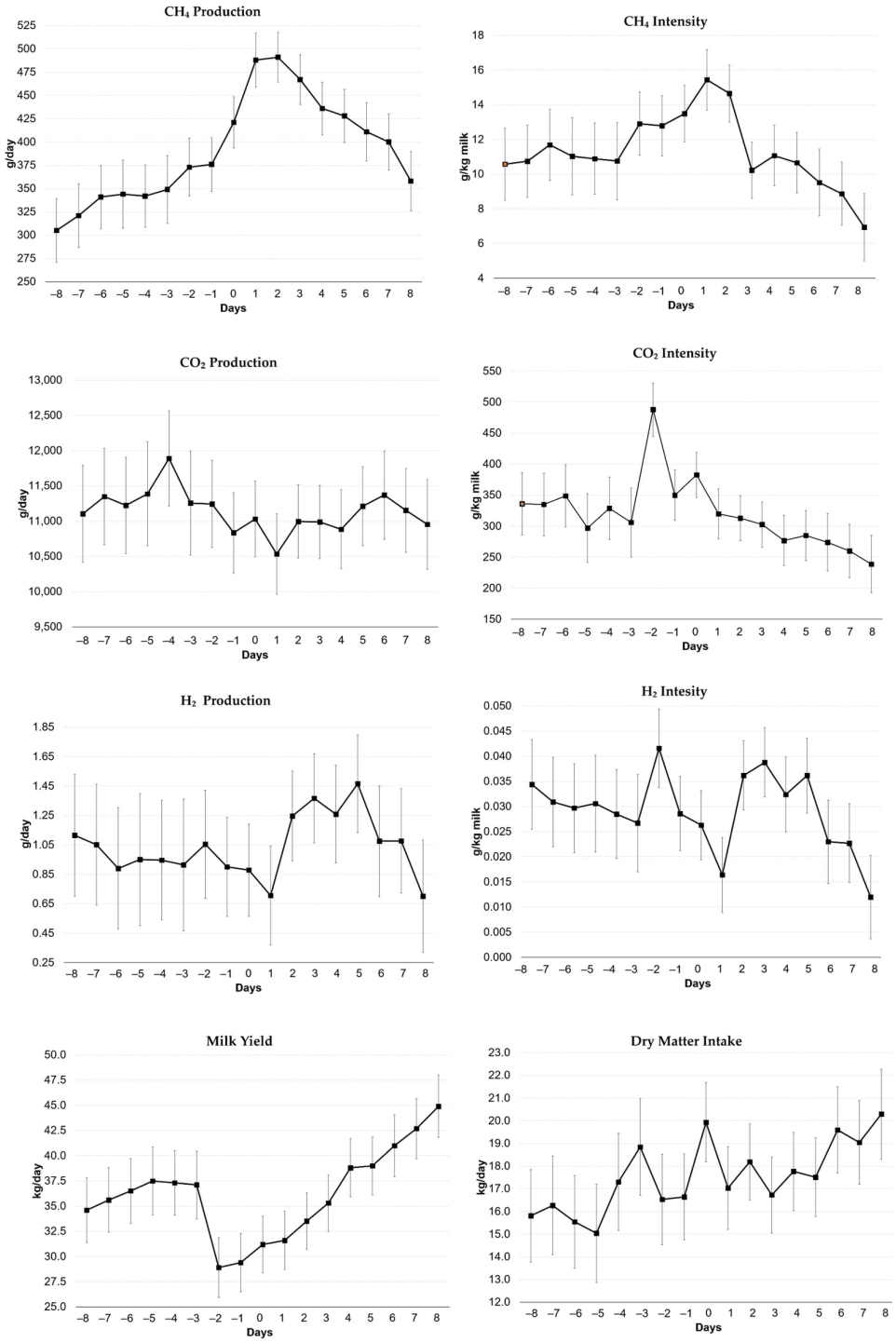



**Figure 4.** Total daily production of methane ($CH_4$; g/day), carbon dioxide ($CO_2$; g/day), dihydrogen ($H_2$; g/day), milk yield (MY; kg/day); dry matter intake (DMI; kg/day), methane intensity ($CH_4$/MY, g/kg), carbon dioxide intensity ($CO_2$/MY, g/kg), and dihydrogen intensity ($H_2$/MY, g/kg) in hyperketonemic cows (HYK, $n = 17$) from 8 days before to 8 days after the first scheduled sampling point at which hyperketonemia was detected. Day 0 represents the first scheduled BHB measurement ≥ 1.0 mmol/L and should not be interpreted as the biological onset of hyperketonemia.

The 95% confidence intervals were estimated using 2000 cow-level cluster bootstrap replicates to account for the correlation between repeated measurements within individual cows. Because the ROC curves and corresponding thresholds were derived and evaluated within the same study population, these findings should be considered exploratory. In particular, the estimated thresholds and their associated sensitivity and specificity require

validation in independent populations before any potential diagnostic application can be considered.

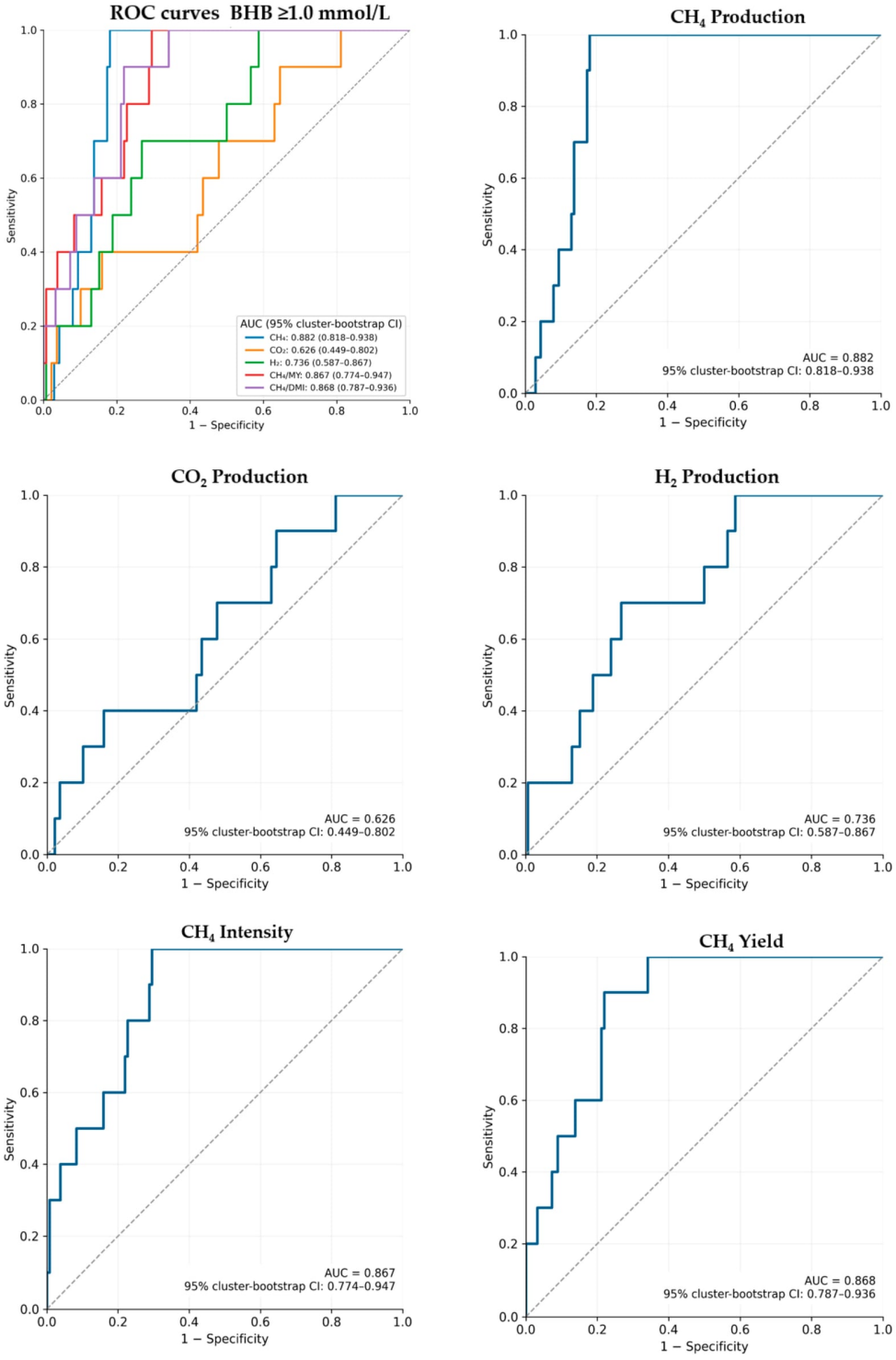



**Figure 5.** Receiver operating characteristic (ROC) curves illustrating the discriminatory ability of $CH_4$, $CO_2$, $H_2$, and $CH_4$ intensity ($CH_4$/MY) and $CH_4$ yield ($CH_4$/DMI) for identifying observations with BHB ≥ 1.0 mmol/L. Areas under the ROC curves (AUCs) were calculated using the available observations for each marker. The corresponding 95% confidence intervals were estimated using 2000 cow-level cluster bootstrap replicates to account for repeated measurements within cows.

## 4. Discussion

Understanding the relationship between metabolic disorders and GHG emissions in dairy cows is crucial for both animal health management and environmental sustainability. Metabolic imbalances, such as hyperketonemia, are associated with substantial changes in energy metabolism and may also be accompanied by alterations in rumen fermentation patterns, potentially affecting enteric gas production [11]. Exploring these interactions provides valuable insights into how physiological disturbances may influence emission dynamics and overall farm efficiency.

The present study confirmed that HYK primarily occurs in early lactation, reflecting the metabolic challenges faced by dairy cows immediately postpartum [38]. Prevalence peaked at 15% at 7 DIM, followed by a decrease to 5% and 7.4% at 14 DIM and 28 DIM, respectively. Similarly, incidence was highest in the first week postpartum (15%) and then decreased over time, reflecting the transient nature of the metabolic imbalance. These patterns are consistent with previous reports indicating that the risk of ketosis is greatest during the early lactation period due to negative energy balance and increased mobilization of body reserves [18,39]. The early peak suggests that the first week postpartum represents a critical window for metabolic monitoring and intervention, supporting the importance of timely detection strategies [40]. Furthermore, the lower incidence observed at one month after calving indicates a progressive recovery or adaptation of the herd's metabolic status, likely due to stabilization of feed intake and milk production [41]. Although the distribution of cows across calendar months did not differ significantly between metabolic groups, this finding does not exclude the possibility of residual environmental or seasonal confounding. Factors such as ambient temperature, humidity, and other time-related variations may have influenced feed intake and the onset of hyperketonemia consequently.

Plasma concentrations of NEFA are widely recognized as sensitive indicators of negative energy balance in early lactation [42]. In the present study, cows in the HYK group exhibited NEFA levels exceeding the commonly used threshold of 0.57 mmol/L [15] during the first two weeks postpartum. At the contrary, mean BHB concentrations rarely exceeded 1 mmol/L. This evidence did not fully reflect the occurrence of HYK in individual cows. Mean values are influenced by healthy animals in each time point. Therefore, BHB should be interpreted in the context of incidence and prevalence of ketosis cases, rather than relying solely on mean concentrations.

Overall, NEFA and BHB should always be considered together, as they provide complementary information. Elevated NEFA reflect intensified mobilization of adipose tissue to meet the energy requirements of lactation, while concomitant increases in BHB indicate enhanced hepatic ketogenesis in response to the energy deficit [43].

The longitudinal phenotype analysis revealed significant differences between cows classified as HYK and CTR during the first month of lactation. Cows in the HYK group exhibited higher daily $CH_4$ production, whereas $CO_2$ and $H_2$ emissions were lower compared with CTR cows. These findings suggest an association between the occurrence of hyperketonemia during early lactation and enteric gas emission patterns, consistent with the potential relationship between metabolic health and environmental performance previously proposed in modelling studies [11]. Importantly, the HYK classification used in the present study represents a cow-level early-lactation metabolic phenotype rather than a continuously hyperketonemic state. Cows were classified as HYK when BHB $\geq$ 1.0 mmol/L was detected at least once during the four scheduled assessments, whereas CTR cows remained below this threshold at all sampling points. Therefore, the higher average $CH_4$ production observed in HYK cows over 3–28 DIM should be interpreted as being associated with cows that experienced at least one detected hyperketonemic event during early lactation, rather than as reflecting continuously elevated BHB concentrations throughout the observation period.

The interpretation of these findings requires caution, particularly because $CH_4$ production in CTR cows was at the lower end of the expected range for high-producing Holstein cows. Although intake-based prediction equations, such as that proposed by [44], would suggest higher $CH_4$ production for cows with comparable DMI, these equations provide population-level estimates and may not fully capture short-term individual variability during early lactation. Moreover, GreenFeed visit frequency, temporal distribution of visits, data-filtering criteria, and calibration procedures were comparable between groups, reducing the likelihood that the observed differences were driven by unequal measurement conditions.

In the primary MY-adjusted models, metabolic phenotype remained significantly associated with absolute $CH_4$, $CO_2$, and $H_2$ emissions. The corresponding sensitivity analyses without MY adjustment yielded comparable estimated between-group differences, supporting the robustness of the observed associations to the inclusion of milk production as a covariate. In particular, the estimated difference in $CH_4$ production between HYK and CTR cows remained similar in the unadjusted (+167.7 g/day) and MY-adjusted (+163.8 g/day) models. The mean daily $CH_4$ emissions observed in HYK cows were within the upper range reported for high-producing Holstein cows under comparable production systems (402.1 ± 25.9 vs. 238–384 g/day) [45–48], whereas the lower values observed in CTR cows should be interpreted with caution. When $CH_4$ emissions were expressed relative to milk yield ($CH_4$ intensity), differences between metabolic groups became more pronounced. These findings reflect an association between metabolic phenotype and methane emission. Similarly, when $CH_4$ emissions were expressed relative to DMI, higher methane yield was observed in HYK cows. Although differences in ruminal fermentation characteristics may contribute to variation in methane production [49], the present study did not include direct measurements of ruminal fermentation, microbial composition, or methanogenic activity. Therefore, the observed differences cannot be attributed to a specific ruminal mechanism.

When $CH_4$ production was evaluated relative to the first detected hyperketonemic event, $CH_4$ production was markedly higher in HYK cows around the first detection of hyperketonemia and during the following assessment period, whereas the difference between groups progressively reduced thereafter. This temporal pattern further supports the interpretation of HYK as a dynamic metabolic condition rather than a persistent metabolic state throughout early lactation. However, these temporal changes must be interpreted cautiously. Because BHB was assessed at approximately weekly intervals, the reference event represents the first scheduled detection of BHB ≥ 1.0 mmol/L rather than the biological onset of hyperketonemia. Therefore, the metabolic alteration may have developed several days before its detection, and transient episodes occurring between scheduled blood samplings may have remained unidentified. Consequently, the present analysis cannot establish whether changes in $CH_4$ production preceded, coincided with, or followed the actual onset of hyperketonemia.

The metabolic disturbances associated with ketosis—such as elevated circulating BHB and shifts in substrate availability—may nevertheless contribute to enhanced methanogenesis [50]. However, these mechanisms could not be directly evaluated in the present study. Previous studies have suggested that changes in feed intake and rumen retention time may influence fermentation pathways and the relative production of volatile fatty acids, potentially modifying the availability of reducing equivalents for methanogenesis [5]. Methanogenic archaea utilize reducing equivalents, primarily in the form of $H_2$, for $CH_4$ formation, and shifts in fermentation pathways could therefore contribute to differences in methane production.

In addition, the increased lipid mobilization accompanying hyperketonemia may be indirectly associated with changes in rumen microbial activity [49]. Lipid metabolites have

been reported to modulate bacterial activity and could consequently influence fermentation pathways and hydrogen dynamics [51,52]. However, volatile fatty acid profiles, ruminal $H_2$ concentrations, microbial community composition, and methanogen abundance were not assessed in the present study. Therefore, these mechanisms should be regarded as biologically plausible hypotheses rather than demonstrated explanations for the higher $CH_4$ production observed in HYK cows.

Meta-analytical data from commercial dairy farms report enteric $CH_4$ emissions in the range of 15–42 g $CH_4$/kg milk (≈700–1200 g $CO_2$e/kg milk using $GWP_{100}$ = 27.2) [53]. Mostert et al., 2018 [19], using a modelling approach, reported that HYK may increase enteric gas emission by approximately 20.9 kg $CO_2$-equivalents per ton of fat- and protein-corrected milk (FPCM). Although these indicators are expressed on different functional bases and are not directly comparable, previous modelling studies provide a broader environmental context for the higher $CH_4$ emissions observed in HYK cows in the present study. However, the present analysis was restricted to measured gas emissions and methane-derived $CO_2$-equivalents and should not be interpreted as an assessment of the carbon footprint of milk production.

The different analytical approaches adopted in the present study provided complementary perspectives. The association between metabolic status and $CH_4$ production appeared more pronounced when evaluated within a longitudinal framework than when considering individual BHB measurements. In the longitudinal analysis, cows classified as HYK based on at least one BHB concentration ≥ 1.0 mmol/L during the scheduled sampling occasions exhibited substantially higher average $CH_4$ emissions throughout the observation period compared with CTR cows. This difference remained evident after adjustment for milk yield and was consistent with the results obtained using a more restrictive BHB threshold of ≥1.2 mmol/L. These findings suggest that the longitudinal classification of cows according to their metabolic profile may capture differences in methane emission patterns extending beyond the specific occasions on which elevated BHB concentrations were detected.

In contrast, when the relationship between metabolic status and $CH_4$ production was investigated using concurrent BHB measurements, the estimated difference in methane emissions was smaller. This difference should be interpreted considering the distinct biological questions addressed by the two analytical approaches. While the concurrent analysis evaluates the association between $CH_4$ emissions and metabolic status at individual sampling occasions, the longitudinal classification aims to capture broader metabolic differences between cows experiencing hyperketonemia during early lactation and those maintaining BHB concentrations below the selected threshold throughout the observation period. This approach is particularly relevant considering that the metabolic alterations associated with the development of hyperketonemia may extend beyond the specific occasions on which circulating BHB exceeds the diagnostic threshold. A single BHB measurement provides information on metabolic status at a specific sampling occasion but may not adequately characterize the broader metabolic trajectory of an individual cow [54]. Therefore, considering the overall emission profile across the first month postpartum provides complementary information on the relationship between metabolic adaptation and enteric gas production. Nevertheless, the longitudinal classification does not establish that increased methane emissions occurred exclusively during periods of active hyperketonemia, nor does it allow the temporal sequence of metabolic and emission changes to be determined.

Additional information was provided by the analyses centered on the first detected hyperketonemic event. The pre- and post-detection comparison identified higher $CH_4$ production following the first detection of elevated BHB concentrations, while the day-by-day analysis revealed significant temporal variation in methane emissions around this reference point. Taken together, these analyses suggest that the relationship between

hyperketonemia and methane emissions cannot be fully characterized through a single analytical perspective.

Nevertheless, the interpretation of pre/post analysis requires caution, as the observed differences may partly reflect the physiological progression of early lactation rather than changes specifically associated with hyperketonemia. Furthermore, in the absence of a corresponding comparison with non-hyperketonemic cows at equivalent DIM, the specific contribution of metabolic status cannot be isolated. Accordingly, these findings should be interpreted as exploratory temporal associations around the first scheduled detection of hyperketonemia rather than as evidence of changes directly caused by the metabolic condition.

The $CO_2$ emissions exhibited a different temporal pattern compared with $CH_4$. Overall, $CO_2$ production was lower in HYK cows than in CTR cows. When evaluated relative to the first detected hyperketonemic event, significant effects of group, time, and their interaction were observed, indicating that the magnitude of the difference between HYK and CTR cows varied over time. As for $CH_4$, these temporal changes should be interpreted relative to the first detection of hyperketonemia rather than its biological onset. However, retrospective analysis integrating measurements over the days surrounding the first detection indicated an overall increase in $CO_2$ emissions following disease detection. This suggests that alterations in $CO_2$ production associated with HYK develop progressively. During ketosis, glucose availability is reduced and the animal shifts toward enhanced lipid mobilization and hepatic ketogenesis, leading to lower rates of carbohydrate oxidation and $CO_2$ production at the cellular level [55]. Differences in ruminal fermentation and hydrogen utilization may also contribute to the divergent patterns observed for $CH_4$ and $CO_2$ [56–58]. However, because rumen fermentation pathways, volatile fatty acid production, and ruminal $H_2$ availability were not directly assessed, the mechanisms underlying the lower $CO_2$ production observed in HYK cows cannot be established from the present data.

When $CH_4$ emissions were expressed as methane-derived $CO_2$-equivalents using the selected $GWP_{100}$ conversion factor, HYK cows showed higher values than CTR cows (10,955 vs. 6631 g $CO_2$e/day, respectively). These values represent only the climate impact attributed to the measured $CH_4$ emissions and should not be interpreted as estimates of the overall carbon footprint or total GHG emissions of the animals or of milk production.

Measured $CO_2$ production was considered separately from methane-derived $CO_2$-equivalents. In particular, the $CO_2$ measured by GreenFeed includes $CO_2$ released through animal respiration, which is predominantly part of the biological carbon cycle and therefore should not be interpreted in the same manner as net fossil-derived $CO_2$ emissions. Accordingly, measured $CO_2$ and methane-derived $CO_2$-equivalents were not combined to calculate a total GHG value.

In ruminants, $H_2$ is a key intermediate of fermentation, serving as a substrate for methanogens to produce $CH_4$. The $CH_4$ production in the rumen also depends on the activity of other microbes that either produce or consume the substrates required by methanogens. Some bacteria generate $H_2$ or methanol during fermentation, while others use $H_2$ and compete with methanogenic archaea [59–61]. Nevertheless, methanogenesis remains the most thermodynamically favorable pathway [62]. In the present study, $H_2$ emissions tended to be lower in HYK cows when $CH_4$ output was elevated. One possible explanation is greater microbial utilization of available $H_2$ for methanogenesis, which could reduce $H_2$ accumulation and subsequent eructation [62]. However, emitted $H_2$ does not provide a direct measurement of ruminal $H_2$ production or utilization, and neither ruminal $H_2$ concentrations nor methanogenic activity were measured in the present study. Therefore, the inverse patterns observed for $H_2$ and $CH_4$ should be considered an association, while the involvement of altered microbial hydrogen utilization remains a hypothesis requiring direct investigation.

Regarding milk production, no clear separation between healthy and diseased cows was observed. This is likely because cows developed ketosis at different time points, which caused the overall average to be influenced by the larger number of healthy animals at each measurement. Consequently, the effect of hyperketonemia on milk production appears diluted when considering the group as a whole. The MY showed a significant increase before and after the first detection of hyperketonemia, as indicated by the aggregated Pre/Post analysis. However, this result should be interpreted in the context of early lactation physiology, as milk production naturally increases over time during the first weeks postpartum [63]. Therefore, the higher post detection mean does not necessarily reflect a beneficial effect of HYK on productivity, but rather the underlying progression of lactation.

When MY was analyzed relative to the first detection of hyperketonemia, a different temporal pattern emerged. A marked reduction was observed approximately two days before the first scheduled detection of hyperketonemia, followed by a gradual recovery in the subsequent days. However, because BHB concentrations were assessed only at approximately weekly intervals, this finding cannot establish whether the reduction in MY preceded the biological onset of hyperketonemia. The subsequent increase in MY may reflect physiological changes associated with advancing lactation [64]. Together, these findings indicate that the day-by-day analysis provides additional information on short-term variations in milk production around the first detection of hyperketonemia that may be masked by broader Pre/Post comparisons. Around the first detection of HYK, increased adipose tissue mobilization leads to a greater release of NEFA into circulation, which can subsequently be incorporated into milk fat synthesis [65]. Consequently, elevated milk fat concentration is frequently reported as a characteristic feature of cows experiencing hyperketonemia and has been proposed as an indirect indicator of altered energy metabolism during the transition period [66].

Analysis of DMI revealed lower feed intake in cows classified as HYK compared with CTR cows. However, the causal relationship between reduced feed intake and hyperketonemia remains difficult to establish. Because the HYK phenotype was defined retrospectively and blood BHB concentrations were measured at approximately weekly intervals, it is not possible to determine whether reduced DMI preceded, accompanied, or followed the biological onset of hyperketonemia. Previous research has suggested that reduced appetite may precede or accompany elevated ketone body concentrations, depending on the severity and timing of the metabolic imbalance [29,67]. Additionally, reduced feed intake during the transition period has been associated with immune activation, as pro-inflammatory cytokines released in response to infections or subclinical inflammation may further suppress appetite [14,68].

When DMI was expressed relative to milk yield (DMI:MY), HYK cows showed lower DMI and a lower DMI ratio than CTR cows. Although these findings indicate differences in the relationship between feed intake and milk production between metabolic groups, the DMI ratio should not be interpreted in isolation as evidence of impaired feed efficiency. Therefore, no conclusion regarding reduced productive efficiency in HYK cows can be drawn from this ratio alone. The concurrent observation of higher $CH_4$ production and lower DMI in HYK cows is noteworthy, as enteric methane production is generally positively associated with feed intake. This finding suggests that differences in DMI alone may not fully explain the observed variation in $CH_4$ emissions between metabolic groups. However, the present observational study does not allow the identification of the underlying biological mechanisms. Potential differences in ruminal fermentation, hydrogen utilization, digesta retention time, or microbial activity remain hypothetical, as these parameters were not directly measured. Therefore, the observed relationship should be interpreted as an

association between metabolic phenotype, feed intake, and methane production, rather than evidence of a direct effect of hyperketonemia on ruminal methanogenesis.

The use of both aggregated and time-resolved statistical approaches allowed a complementary interpretation of the effects of HYK on gas emissions and productive traits. The comparison between before and after the first detection of hyperketonemia periods provided a synthetic evaluation of overall changes observed around the first detection of hyperketonemia, whereas the day-by-day analysis captured short-term and transient responses occurring near detection. In some cases, variables exhibited significant differences in the temporal analysis but not in the Pre/Post comparison, or vice versa. This reflects the fact that averaging measurements over multiple days may attenuate or mask acute fluctuations that are biologically relevant but temporally limited. Conversely, effects that are sufficiently pronounced and sustained over time, such as the increase in $CH_4$ emissions and DMI observed after the first detection, remain detectable even when responses are averaged across broader periods. Together, these findings highlight the importance of integrating both approaches to fully characterize the magnitude and dynamics of metabolic disturbances associated with HYK.

The exploratory ROC analysis suggested that enteric gas emissions may provide information on concurrent metabolic status in early-lactation dairy cows, defined according to blood BHB concentrations $\geq 1.0$ mmol/L. Among the evaluated variables, absolute $CH_4$ production showed the highest apparent discriminatory ability, with a data-derived exploratory threshold of $\geq$336.99 g/day. The $CH_4$ intensity and $CH_4$ yield also demonstrated good apparent discriminatory ability, with AUC values of 0.867 and 0.868, respectively. These findings are consistent with the associations observed in the longitudinal analyses and suggest that methane-related variables may reflect metabolic alterations associated with hyperketonemia. However, these results should be regarded as exploratory and hypothesis-generating rather than evidence of established diagnostic performance.

In contrast, $CO_2$ production showed limited discriminatory ability, whereas $H_2$ production demonstrated moderate apparent discriminatory ability. The relatively lower discriminatory performance of these gases compared with methane-related variables may reflect their involvement in multiple physiological and microbial pathways, as previously discussed. Their emissions may therefore be influenced by factors beyond the metabolic alterations associated with hyperketonemia, potentially limiting their specificity as indicators of metabolic status. Nevertheless, the findings for $H_2$ suggest that its potential relationship with concurrent BHB status warrants further investigation.

Importantly, the ROC analysis was conducted within a single herd, with a limited number of BHB-positive sampling occasions and BHB measurements collected at approximately weekly intervals. Accordingly, the reported AUC values, sensitivity, and specificity represent apparent performance within the present study population. In particular, the exploratory $CH_4$ threshold should not be interpreted as a generalizable diagnostic cutoff, as enteric methane production is influenced by multiple animal-, diet-, management-, and environment-related factors. Further studies involving larger and independent populations, more frequent metabolic assessments, and different herd management conditions are needed to investigate the potential application of automated methane monitoring as a complementary indicator of metabolic status in dairy cows.

The present study provides novel evidence of the relationship between metabolic health and enteric gas emissions; some limitations must be considered when interpreting the outcomes. The use of a GreenFeed system, although validated for on-farm measurement of gases eliminated by eructation, differs from respiration chamber techniques, which allow for complete capture of emitted gases under fully controlled conditions. Moreover, rumen fermentation parameters, volatile fatty acid profiles, ruminal $H_2$ concentrations, microbial

community composition, and methanogen abundance were not directly assessed in the present study. Consequently, the biological mechanisms underlying the observed associations between hyperketonemia and gas emissions cannot be established from the present data. The potential involvement of altered fermentation pathways, hydrogen availability and utilization, and methanogenic activity should therefore be regarded as biologically plausible hypotheses based on previous literature rather than mechanisms demonstrated by the present study. Future studies combining longitudinal measurements of metabolic status and gas emissions with direct characterization of rumen fermentation, volatile fatty acid profiles, and microbial communities are needed to clarify the mechanisms underlying these associations.

Another limitation concerns the measurement of blood BHB, which was not performed daily. As a result, the precise timing of HYK onset may have been underestimated.

The present study was not designed as a life-cycle assessment and did not quantify all sources contributing to the carbon footprint of milk production. Methane-derived $CO_2$-equivalents were calculated solely to express the climate impact associated with measured $CH_4$ emissions using the selected $GWP_{100}$ factor. Measured $CO_2$ emissions were considered separately and were not combined with methane-derived $CO_2$-equivalents to estimate total GHG emissions or an overall carbon footprint.

Finally, the monitoring period was restricted to the first month of lactation. Extending both emission and metabolic monitoring across the entire transition period and full lactation would provide a more comprehensive understanding of the relationships among metabolic status, rumen function, production efficiency, and measured gas emissions in dairy cows.

## 5. Conclusions

The present study identified an association between hyperketonemia and enteric gas emissions in early-lactation dairy cows. Cows classified as HYK exhibited higher $CH_4$ emissions, both in absolute terms and relative to milk yield and dry matter intake, despite having lower feed intake. Conversely, $CO_2$ and $H_2$ emissions were lower in HYK cows. The complementary analytical approaches highlighted different aspects of the relationship between metabolic status and methane production, with more pronounced differences observed when considering the longitudinal metabolic phenotype than when evaluating concurrent BHB concentrations. These findings suggest that methane emission patterns may reflect broader physiological and metabolic characteristics during early lactation rather than exclusively the circulating BHB concentration measured at individual time points. However, the observational nature of the study does not allow causal relationships or underlying ruminal mechanisms to be established. Further investigations involving larger populations, more frequent metabolic assessments, and direct measurements of rumen fermentation are needed to clarify these associations and evaluate their potential implications for metabolic health management and the environmental sustainability of dairy production.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani16233790/s1, Table S1: Summary of GreenFeed visitation frequency in control (CTR) and hyperketonemic (HYK) dairy cows during the experimental period (3–28 DIM); Table S2: Comparison of milk-yield-adjusted linear mixed-effects models for absolute methane ($CH_4$), carbon dioxide ($CO_2$), and hydrogen ($H_2$) emissions using all available cow-days (≥1 valid GreenFeed visit/day) and a restricted dataset including only cow-days with ≥2 valid GreenFeed visits/day.

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G.S., M.B. and M.G.; final approval of original draft: E.T., F.P.-C., F.A. and M.G.; founding: E.F. and A.L. All authors have read and agreed to the published version of the manuscript.

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**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are not publicly available since they are still under analysis for further publications.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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