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Methane, Nitrous Oxide, and Ammonia Emissions on Dairy Farms in Spain with or without Bio-Activator Treatment

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Abstract: Intensive livestock farming substantially impacts the environment, especially farm and slurry management. Slurries are significant sources of greenhouse gases and ammonia. The present study was conducted in an intensive livestock production system in Galicia, Spain. The measurements were taken at six different farms in that region along with one control using common management practices in Galicia without the addition of a bio-activator. This study aimed to quantify GHGs and NH_3 fluxes and their reductions during slurry treatment using a dynamic chamber through FTIR analysis and to examine the potential of usage of bio-activators for slurry management. In addition, gas concentrations were measured at the barns and compared with their slurry management and architectural volume to obtain influences on their management and the architectural volume of the barns. Additionally, the effects of using a bio-activator in the barns inside the facility areas were addressed. Moreover, qPCR analysis was conducted to understand the correlations between syncoms and methanogen populations when a bio-activator is added to the slurry with at least a 30% reduction in methanogenic populations. The outcomes suggest encouraging results for GHG reductions in the livestock sector, giving farmers future options for climate change mitigation among their standard practices.

Keywords: emissions; slurry; syncoms; bio-activator

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1. Introduction

Livestock is a common source of methane (CH_4), nitrous oxide (N_2O), and ammonia (NH_3) emissions [1], where CH_4 and N_2O are powerful greenhouse gases (GHGs), and agriculture is the primary source in Europe contributing to global warming [2]. During livestock and rearing practices, the storage and management of slurry (i.e., a mixture of feces, urine, sand bedding material, and water) are essential factors to consider [3], because during storage, GHGs are formed. CH_4 emissions occur in response to organic matter degradation under anaerobic conditions. N_2O is formed by the denitrification of nitrate (NO_3^-) and the nitrification of ammonium (NH_4^+) processes [4]. NH_3 is not a GHG; nevertheless, it has multiple negative environmental impacts due to the potential pathway of nitrogen (*N*) loss through NH_3 volatilization in the air, water, and soil [5]. Consequently, the mismanagement of cattle slurry generates pollution in the form of GHG and NH_3, accounting together with enteric fermentation for 80% of global CH_4 emissions from agriculture [6]. For example, the share of the contribution of these pollutants for livestock systems are CH_4, 21% N_2O, and 75% NH_3 to total agricultural emissions in the European Union according to the UNFCC (United Nations Framework Convention on

Climate Change) in 2016. Therefore, sustainable solutions for better management practices during slurry storage must be considered [6].

Milk is one of the most produced foods worldwide, and Spain currently ranks seventh among European milk producers, with 5% of the total European Union milk production [7]. Galicia generates between 36 and 40% of the total milk production in the country [8]. Slurry is usually stored under three alternatives conditions during intensive livestock production [9]. One method is to store the slurry under an aerobic process, where aerobic bacteria operate under sufficient oxygen conditions. The second option is under an oxygen-free system using so-called anaerobic microorganisms. The third alternative is facultative storage, which operates under aerobic and anaerobic conditions with facultative bacteria [10]. In Spain, it is mandatory to have a roof covering, and the storage tank must have the capacity to store slurry for a minimum of 6 months, and it can only be added to the field at certain times of the year, according the Royal Decree 2224, Law 1/1995, on environmental protection in Galicia, and Council Resolution 97/C 76/01.

Soil is a matrix of mineral and organic compounds where complex chemical, physical, and biological interactions occur. Therefore, its study was focused on physical and chemical characteristics for a very long time. The role of microorganisms and the ecology of their populations have become relevant in the study of soil dynamics and properties [11,12]. The analysis of microorganism communities, particularly the rhizosphere, has revealed the complexity of the structure and the strengthened relationships among all soil organisms [13–15]. Metagenomics, proteomics, transcriptomics, and bioinformatics techniques are the basis of these analyses. These allow a description of some communities in different soil types, crops, and various environmental or management conditions [16].

The different species of microorganisms and how they relate to each other, to plants, and to fauna and the distinct differences that were found with the soil aggregates are decisive for the proper functioning of the different biogeochemical cycles and the movement of the elements from the mineral fraction to the biomass and vice versa [17]. Numerous studies have pointed out the importance of fungi and bacteria in cycles such as carbon and nitrogen [18–21]. For example, certain farms are currently using biological activators to improve dairy slurry management in Spain. Biological activator products act as a microbial inoculation, where the microorganisms are more competitive than those initially present in the manure, accelerating the biological oxidation process of organic matter degradation [22]. Furthermore, synthetic communities of microorganisms (i.e., syncoms) have been described to modify the effect of different crops' soil health and productivity [23]. Similarly, some of these syncoms, such as efficient microorganisms or bio-activators, have been used to reduce foul odors in composting processes and improve the physical characteristics of organic amendments for agricultural use [24–26]. In this way, the possibility of using products that combine different species of microorganisms to modify the structure of the soil community and intervene in the cycles of different chemical elements is raised, reducing some emissions into the atmosphere along the way [27]. This new approach constitutes an element of integration between the chemical and biological components of the soil.

This study investigated the efficacy of GHG reduction from dairy cattle slurry in Spain using a bio-activator. Moreover, a correlation between microbial community and methanogen behavior through quantitative PCR (qPCR) analysis using a bio-activator in the slurry was addressed. In addition, emission concentrations at the level of the barn, according to the management of each farm using bio-activators in the halls to obtain the behavior of emissions due to the architectural volume differences and management among the farms, were measured. The objectives of our field experiment were to evaluate the effects of the bio-activator (i) on slurry storage, (ii) on methanogens activity in the slurry, and (iii) on the barns for emission concentration quantification.

2. Materials and Methods

2.1. Research Site and Description

The study was performed in Mazaricos 42.9399° N, 8.9923° W, Galicia, Spain. Galicia is a region of Spain located in the northwest of the country. Six different dairy farms in the region were selected for the study. The conditions for the control farm were not using a commercial bio-activator. The other five farms had been using the product for at least a year, following the application instructions recommended by the manufacturer. In order to maintain the privacy of the farmers, the farms are named from F1 to F6, with F1 being the control during this study.

The following Table 1 summarizes the status quo of the farms taken into consideration for the study. The selection of the farms was based on the similarities among them to avoid more external factors influencing the results. Farmers provided the corresponding information on animal nutrition, additional inputs, and slurry storage time. The volume of the slurry was calculated by measuring the area of storage and the amount of slurry inside the tanks. A sample of a 90 cm depth was taken to measure the density of the slurries. Temperature and pH were directly measured in the slurry using a Milwaukee Instrument PH55, (Milwaukee Instrument, Rocky Mount, NC USA). The dairy cattle were counted during the measurements, and to corroborate the numbers, the farmers provided the number of cows per farm for this study.

Table 1. Status of the farms and properties of the slurries.

Barn					
Farms	***N* Dairy Cattle**	**Animal Nutrition**	**Type of Farming**	**Storage Slurry Weeks**	**Bedding Material**
F1	150	30–40% Corn Silage 20% Straw 11–13% Mixed Forage 15–20% Dry Organic Compounds	Intensive	4	Sand
F2	130			3	
F3	120			4	
F4	130			4	
F5	100			3	
F6	130			4	

Slurry Tank							
Farms	**Temperature °C**	**pH**	**Density kg/m^3**	**Additional Inputs**	**Appearance**	**Type**	**Volume Slurry m^3**
F1	16.8	6.9	1050	Disinfectants, Drug Residues, Hormones, Formalin, Fungicide, Copper, Cow Milking	Dense	Semi-Open	907.2
F2	16.6	6.8	1050		Liquid		1500
F3	14.8	6.7	1020		Liquid		750
F4	16.7	6.9	1040		Liquid		665
F5	14	6.9	1070		Liquid		1035
F6	15.5	6.9	1020		Semi-Dense		1776

2.2. Gas Measurements and Sampling Procedure

Six farms were investigated with five of them applying the bio-activator. One farm was used as a control (F1). The emission fluxes during the study while using a bio-activator were compared to the control farm that did not use a bio-activator to determine a depletion or increase in emissions when the product was used. The sampling campaign was conducted in November 2021 at all the farm facilities during the mornings and afternoons (between 8:00 and 17:00) without interrupting the daily farm activities. Emission measurements were taken prior to oxygenation and movement of slurry inside the tank. Before the measurements in the slurry tanks, an estimation was made at constant concentrations until

the gas was in equilibrium. An appropriate mix of air inside the chamber was reached by the airflow between the FTIR and the chamber. It was tested for air-tightness air losses and calibrated according to Gasmet recommendations [28]. The measurements in the slurry tank were taken daily on each farm at 20 s intervals for 60 min, with a total of at least 180 observations during each measurement at the farm. The gas fluxes were determined using an open-dynamic chamber method. The chamber was made floatable in a sealed cylindrical shape with a vent for pressure change avoidance [29]. Inside the hood, the ambient air was flushed with a constant flow, and the gas concentration was quantified with a background calibration. Its difference with the off-gas stream coming from the hood in equilibrium concentration was measured with the Fourier transform infrared FTIR GT500 Terra from Gasmet Technologies GmbH, Karlsruhe, Germany. The air exchange started once the sampling hood was placed, and the equilibrium was reached when the outgas flow was equal to the gas flux discharge from the measurement surface. The FTIR can identify up to 50 different compounds, with a detection limit of <3 times the spectral baseline. With this device, CH_4, N_2O, and NH_3 were measured. According to Gasmet Technologies, its principle is based on infrared radiation (IR). IR is related to infrared molecules, and it has the effect of causing vibrations (bending or stretching) in the molecule. A molecule will absorb IR radiation and will experience vibrations among its atoms. The energy and, thus, the wavelength of the radiation requires the atom's bondage. The concentration of the components can be measured with FTIR when the light is traveling through the material. The amount of absorption depends on the concentration of the material. All active IR compounds have different spectra; thus, each component can be identified, since the FTIR possesses known compounds in a gas library with different concentrations. The gas equilibrium concentrations differences were interpolated with the chamber, ambient, and flow rate divided by the slurry area covered in the chamber, obtaining the emission fluxes.

Prior to the barn measurements, the background concentration outside the barn was measured for at least 30 min. Then, the sampling was performed inside the barn including the halls where the animals were living. The concentrations of CH_4, N_2O, and NH_3 were continuously measured. Triplicate measurements were taken at each point to obtain only gas concentrations within the barn moving towards a 1 m distance, taking equidistant points within the measurement area of the barn. The floor was made of concrete and was cleaned at least three times daily with an automated cleaning robot, and the measurements were taken before the robot initiated a cleaning cycle on the floor's surface. The walking direction, farm orientation, passive airflow through open space sources, such as windows or doors, and architectural volume were considered. The number of open doors and windows varied on some farms; for example, F2 and F3 had just two doors on the sides, whereas F6 had the highest number with seven doors—three on two sides and one frontal door on the other walls—and F1–F6 possessed three open windows on the sides. Wind direction was also necessary to understand how the movements of the gases occurred inside the barn. The wind speed was between 3 and 5 m/s among all the farms. The wind direction was in the south direction for F1 and F5, southwest for F6, west for F4, and north for F2.

2.3. Flux and Emission Rates

The chamber method allows for diffusivity to measure the concentration over time and calculate the flux quantification. By geometric calculations, the volume of gas inside the chamber was calculated as follows and is represented in Figure 1.

$$\mathrm{Vg} = \frac{\pi}{3} \times (H - Hb) \times \left(R'^2 + r'^2 + R' \times r'\right) \quad (1)$$

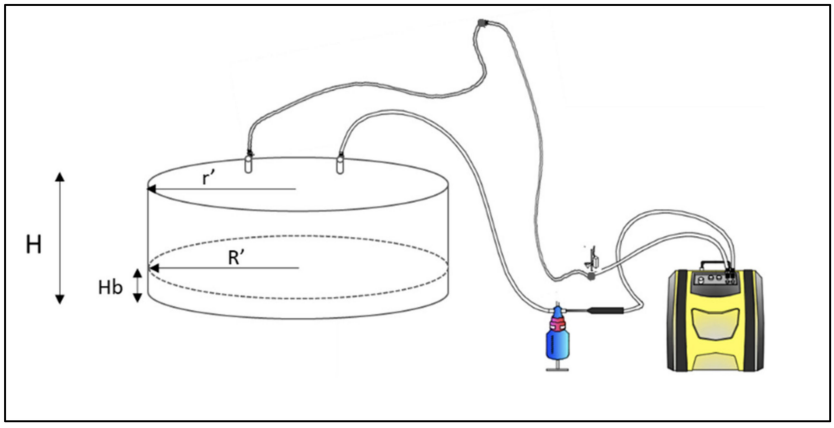

Figure 1. Setup for the gas measurements using a dynamic chamber.

H and *Hb* correspond to the total height and the inside slurry height of the chamber, while R' is the lower radius on the surface of the slurry, and r is the upper radius of the chamber.

Some of the factors considered for the flux calculations were the chamber area, flow volume, and volume. Finally, the fluxes were calculated in g $\times$ m^{-2} $\times$ h^{-1} for CH_4 and NH_3 and in mg $\times$ m^{-2} $\times$ h^{-1} for N_2O with the following equations:

$$Q_{gas} = \frac{V}{A} \times \frac{\Delta c}{\Delta t} \times \frac{Mgas}{22.4} \times \frac{273}{K} \quad (2)$$

Q_{gas} = Flux of the gas (g $\times$ m^{-2} $\times$ h^{-1} or mg $\times$ m^{-2} $\times$ h^{-1});
V = Volume of the chamber (m^3);
A = Base area of the chamber (m^2);
($\Delta c/\Delta t$) = Rate of decrease/increase in the gas concentration (mg $\times$ m^{-3});
M = Molar weight of the gas;
K = Temperature of the air in *K* inside the gas.

The emission factor was calculated as the mass ratio of *gas* emitted to initial fresh matter mass (g $\times$ kg^{-1} slurry).

$$EF_{gas} = \frac{Qgas \times t_{treat} \times A_{treat}}{m_{treat}} \quad (3)$$

EF_{gas} is the emission factor of the gas related to the mass of slurry treated (g kg^{-1} slurry); q_{gas} is the emission rate of the gas (mg $\times$ m^{-2} $\times$ h^{-1}); t_{treat} is the duration (time) of treatment (h); A_{treat} is the area of treatment (surface area of the emission) (m^2); m_{treat} is the mass of the slurry (kg).

2.4. Bio-Activator

The commercial bio-activator used in the study was Bioprana™ (Lugo, Spain), which accelerates the slurry decomposition process with microorganisms, eliminates foul odors, facilitates handling, and improves its fertilizing power. For the activation of the bio-activator, 20 L of the bio-activator (Bioprana™) was diluted in 1000 L of water without chlorine or hydrogen peroxide, supplemented with 2 L of whole milk, and kept protected from direct sunlight. These conditions promoted the activation of the product, allowing maximum efficiency in its application. A dose of 5 L per m^3 of the slurry was applied daily until the end of the experiment. The product was a mixture of bacteria, yeasts, and actinomycetes, among other microorganisms, with a chemical oxygen demand of COD = 2534 mg O_2 $\times$ L^{-1} and 69,119 mg O_2 $\times$ L^{-1} and a biochemical oxygen demand for five days of BOD = 500 mg O_2 $\times$ L^{-1} and 7500 mg O_2 $\times$ L^{-1} when the product was activated in a concentrated form, respectively.

2.5. Microbiology in Slurry Samples

Slurry samples were taken from the slurry tank of the F6 farm at a 65 cm depth. This farm was selected because no bio-activator was used there before the start of the study. These samples were taken before the microorganism treatment (Sample 0) and weekly for four weeks. Once obtained, samples were kept on ice until qPCR analyses were carried out. These qPCR analyses were carried out by AllGenetics & Biology SL [30].

2.6. Quantitative PCR Analysis

A total of 5 slurry samples were received at AllGenetics on 26 October 2021. DNA was isolated upon arrival at the laboratory using 250 mg of each sample and the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany), strictly following the manufacturer's instructions. DNA was resuspended in a final volume of 50 µL. An extraction blank was included in every DNA extraction round and treated as a regular sample to check for cross-contamination. Methanogen community quantification was performed by quantitative PCR (qPCR), targeting the mcrA gene with primers Mlas-mod-F (5′ GGYGGTGTMGGDTTCACMCARTA 3′) described by Angel, Claus, and Conrad [31] and modified by Steinberg as mcrA-R (5′ CGTTCATBGCGTAGTTVGGRTAGT 3′) [32]. The qPCR was performed with the nonspecific fluorophore SYBR Green included in the NZYSupreme qPCR Green Master Mix (2x), ROX plus (NZYTech, Lisbon, Portugal).

The first treated sample was used to amplify the mcrA gene with Mlsa-mod-F and mcrA-R primers. PCRs were carried out in a final volume of 25 µL, containing 2.5 µL of template DNA, 0.5 µM of the primers, 12.5 µL of Supreme NZYTaq 2x Green Master Mix (NZYTech), and ultrapure sr up to 25 µL. The reaction mixture was incubated as follows: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 53 °C for 30 s, 72 °C for 45 s, and a final extension step at 72 °C for 5 min. This PCR product was purified and used to generate the standard curve in the qPCR experiment. The qPCRs were carried out in a final volume of 20 µL, containing 10 µL of NZYSupreme qPCR Green Master Mix (2x), ROX plus (NZYTech), 2 µL of template DNA, and 0.4 µM of the primers. The reaction mixture was incubated as follows: an initial incubation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 53 °C for 1 min, and extension at 60 °C for 1 min. Negative qPCR controls that contained no DNA were included to check for cross-contamination. qPCR was performed in triplicate on each sample and control.

2.7. Statistical Analysis

A one-way analysis of variance (ANOVA) test was carried out for the emissions measurements. A significance level of $p < 0.05$ for slurry emissions was used for all mean values. In addition, the Tukey HSD test ($\alpha = 0.05$) was used to assess significant differences between treatment means at a 5% probability level. Moreover, for the qPCR results, analysis of data was performed by Microsoft Excel (Microsoft, Redmond, WA, USA). Student's *t*-test was performed to analyze the significance of the matched-pairs data. Again, $p < 0.05$ served as the confidence interval for separating the significantly different means.

3. Results and Discussion

3.1. Slurry Emission Fluxes

Each farm stored the slurry in tanks where it performed a series of reactions, activities, and gas exchanges within the atmosphere. The amount of manure produced and the amount that decomposes anaerobically are the two most important factors impacting CH_4 emissions [33]. Weather conditions had no direct impact on the outcomes, since the tanks were semi-open. Despite this, no rain or particularly strong winds were reported throughout the measurements. A 10–15 cm thick surface crust of fibrous organic material was seen over the slurry in the control (F1) tank surface; however, little or no surface crust was seen in the remainder of the farms using the commercial product.

FTIR spectroscopy and chamber methods have proven to be valuable methods for measuring and quantifying CH_4, N_2O, and NH_4 emissions [34–37]. Therefore, the development

and implementation of this methodology was proposed. Table 2 reveals the results of the emission fluxes and follows their emissions factors for CH_4, N_2O, and NH_3. In the slurries, they were reduced by 42% to 86% when compared to regular farm management. The presence of anaerobic microsites inside the slurry and a lack of aeration is a possibility of its emissions in the control, which models the most common slurry management in Spanish farms [38]. On the other hand, GHG reductions were seen in farms F2–F6. This might be due to the absence of crust formation in the tanks due to the usage of a bio-activator [39].

Furthermore, the surface crust may act as a barrier to CH_4 emissions in anoxic slurry, but when the tank is stirred, all GHG gases are released [39,40]. Even while research on syncoms in cattle slurry is scarce, they exhibited a positive impact [41]. Methanogens are suppressed by the release of enzymes, organic acids, and amino acids by these highly concentrated and active populations of microorganisms [42]. Likewise, they provide conditions such as a high degree of homogeneity, higher mass transfer, and better mobilization in the slurry [43]. These conditions can be accomplished by replacing undesired methanogens with beneficial microorganisms [44].

Table 2. Emission fluxes and emission factors in the slurries.

Item	Farms (n = 3)							
	1	2	3	4	5	6	SEM *	p-Value
Emission Fluxes, g × m^{-2} × h^{-1}								
CH_4	103.41	39.4	22.49	38.91	37.65	14.51	1.10	0.31
N_2O	2.99×10^{-2}	3.00×10^{-4}	3.06×10^{-3}	3.00×10^{-4}	6.12×10^{-3}	3.06×10^{-3}	0.001	0.33
NH_3	0.13	0.25	0.29	0.32	0.43	0.15	0.01	0.16
Emission Factor, g × kg^{-1} Slurry								
CH_4	139.03	70.44	39.06	57.25	54.95	23.63	2.24	0.43
N_2O	4.02×10^{-2}	5.00×10^{-4}	5.31×10^{-3}	5.00×10^{-4}	8.93×10^{-3}	4.98×10^{-3}	0.001	0.33
NH_3	0.17	0.44	0.51	0.46	0.62	0.24	0.02	0.16

* SEM: standard error of the mean values. Since only the activated product was applied to the slurry, the COD of the slurry was frequently high, with an average value of approximately 73,000 mg $O_2 \times L^{-1}$ [45]. As a result, the increase in organic load attributable to the product was insignificant, implying that the total COD of the slurry product mixture was unaffected, since the commercial product was not contributing to the increase in the COD inside the tank.

Furthermore, if the volume applied was less than 0.5 percent of the entire volume of slurry, it did not imply a dilution of the COD. As a result, while the change in the COD total was only 0.48 percent, this small change does not represent a significant change in the slurry's natural environment, which could alter the anaerobic conditions that are typically generated due to the high demand for oxygen required for organic matter oxidation. The microbial shift that occurs in the slurry is important. This indicates that the product has no effect on the physicochemical environment. As a result, methanogens can survive in vast quantities of COD, which is the primary cause of an increase in CH_4 emissions [46].

Overall, the highest CH_4 emission flux was found in the control with 103.41 g × m^{-2} × h^{-1}, and the lowest emission detected was recorded in F6 with 14.51 g × m^{-2} × h^{-1}. On the other hand, N_2O reported a reduction of at least 50% in the farms after adding the commercial product. The emissions fluctuated between 29.91 mg × m^{-2} × h^{-1} and 0.03 mg × m^{-2} × h^{-1}. The results show similarities with other studies regarding fluxes on N_2O [47]. The reductions might be related to the nitrification and denitrification process occurring in the interfaces between air liquid and filled-air pores. Researchers have found similar behavior in N_2O reduction when straw and crust were not found in slurry tanks [48].

For the results regarding NH_3 fluxes, the lowest value was found in F1 at 0.13 12 g × m^{-2} × h^{-1} and the highest at 0.43 g × m^{-2} × h^{-1} in F5. Similar results were reported in 711 cases studied, where the baseline of NH_3 emissions was 0.12 g × m^{-2} × h^{-1} in cattle slurry tanks [33]. On the other hand, F6 presented the lowest emission among all the farms tested. One of the reasons could be the formation of a thin layer on the surface

of some tank areas. This layer may cause crust formation and interfere with measurements. Furthermore, methodological reasons and physical vectors prevented accurate measurement in F6, resulting in slightly different results than the rest of the farms using the bio-activator. According to the physical appearance of the crust inside the slurry at that farm, as shown in Table 1, the crust was semi-dense. Furthermore, some external factors to the slurry were observed such as postpartum residues and a high quantity of sand bedding material. These observations may make taking the measurements in the slurry properly more difficult, obtaining different values compared to the rest of the farms using the bio-activator.

Research studies found a connection between the crust and the emissions, stating that the emissions are enhanced when the surface does not have a thick crust; therefore, NH_3 oxidizing bacteria could not contribute to the nitrification process in the slurry [49]. Instead, NH_3 emissions are produced due to volatilization and formed during the biodegradation of organic matter [50]. Their formation occurs through a microbial breakdown of nitrogen and organic compounds, depending on the equilibrium predominantly NH_3/NH_4^+ changing to NH_4^+. Different gases are emitted on the surface by diffusion and convection, where particles of air and liquid enhance the movement of the compounds to the surface [51]. During this interface, the gases are passed through liquid by convection, and the transport of the liquid phase will depend on the turbulence and the organic matter of the slurry developing natural crust formation, creating a barrier to the gas molecules between the air and the liquid phase [52]. Therefore, the increase in emissions is due to the increment of available NH_4 in the tank. Another possibility stated by other researchers is that this effect might be attributed to the additives or microorganisms when added into the slurry, resulting in a more porous crust, increasing the NH_3 production [53].

The Intergovernmental Panel on Climate Change (IPCC) states the standard emission factors. However, the results and their ranges are extensive, depending on the type of manure and management, duration of storage, aeration, the addition of microorganisms, or external additives involved [54,55]. Currently, no emissions factors are specifically referenced with syncom usage in cattle slurry treatment. Therefore, the data obtained in this study are of great importance to contribute to future inventories. During this study, emissions factors were given in $kg \times kg^{-1}$ of slurry. The highest emission factor was found in F1 as a control for CH_4 and N_2O. On the other hand, the lowest emission factor for NH_3 was found in the control among the usage of the commercial product in the slurry treatment. These values corroborate the effect of syncom in cattle slurry treatments for reducing GHG gases and future assessments in the dairy farms and slurry management.

3.2. Bio-Activator and Population of Methanogens

StepOne (Applied Biosystems, Waltham, MA USA) software was used to register the intensity of the fluorescence emitted by the fluorophore at each cycle of the qPCR reaction for each sample to estimate their Cq value (quantification cycle). The Cq value is the PCR cycle number at which the accumulating PCR products' fluorescence reaches a pre-established threshold. It is inversely related to the starting amount of target DNA. Estimates of the Cq values for each sample are given in Table 3. In addition, the copy number of the mcrA gene using the amplicon size information obtained on the agarose gel, which was around 400 bp, was also estimated [56].

A total of five 10-fold dilution series of known mcrA gene copy numbers from week treatment 1 (ranging from 0.27 ng to 2.68×10^{-5} ng) were used to establish the standard curve and assess the reaction efficiency. These dilutions were also performed in triplicate.

Table 3. Results of the detection and quantification of the mcrA gene qPCR.

Analysis of Methanogen Communities Using qPCR in Slurry Samples					
Treatment Weeks	**Cq Mean ***	**Cq SD ****	**Quantity Mean ng in 50 µL**	**mcrA Copy Number in 50 µL *****	**Cq Threshold**
0	12.89	0.05	3.12×10^{-2}	7.11×10^{7}	2.66
1	12.90	0.05	3.11×10^{-2}	7.09×10^{7}	2.66
2	13.79	0.10	1.98×10^{-2}	4.51×10^{7}	2.66
3	14.87	0.13	1.15×10^{-2}	2.63×10^{7}	2.66
4	13.88	0.21	1.90×10^{-2}	4.33×10^{7}	2.66

* Cq Mean: average Cq values for $n = 3$. The standard deviation of the Cq value $n = 3$. mcrA copy number per 50 µL of DNA sample. SD ** Standard Deviation of the mean. Cq *** value $n = 3$. mcrA copy number per 50 µL of DNA sample.

The standard curve obtained slope of −4.58, a Y-inter value of 4.18, an R^2 of 0.9998, and an amplification efficiency calculated from the standard curve slope of 65.3% (Figure 2). The amplification efficiency obtained was low; therefore, further studies are necessary to improve this. Nevertheless, this value is enough to ensure an effect on the methanogen population after applying the biological activator.

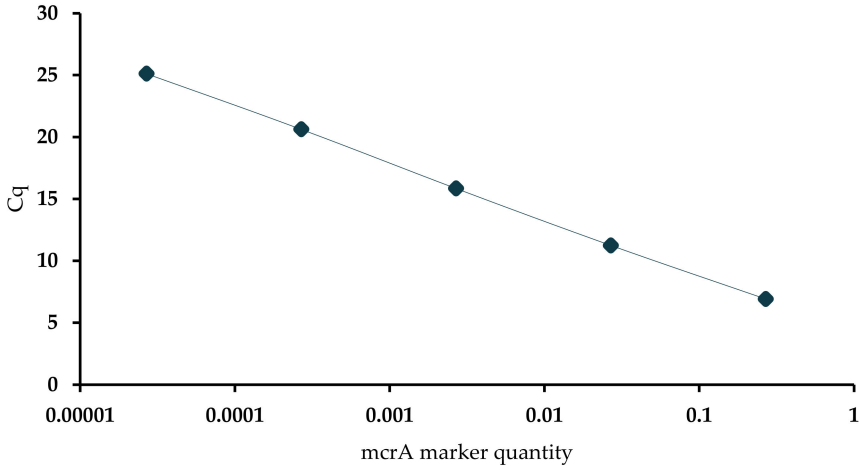

Figure 2. Standard curve produced by plotting the known quantity for the five 10-fold dilution series of the first treated sample for the mcrA marker versus their Cq values.

In the study carried out over four weeks of treatment, it was observed that the biological activator needed one week to begin to have a significant effect on the population of methanogens ($p > 0.05$) between sample 0 and 1. It reached its maximum reduction (highest Cq) at three weeks, with this value being lower than all the others ($p < 0.05$); in the fourth week, a rebound in the population was observed (lower Cq), although the result was still significatively lower than in week 0 ($p < 0.05$) (Figure 3). There were no differences between weeks 2 and 4 ($p > 0.05$) Moreover, Table 4 shows the statistical analysis of the results presented in Figure 3.

Table 4. Statistical analysis results of the comparisons between weeks of treatment.

Treatment Weeks	**t**	***p*-Value**
0 vs. 1	−0.15	0.89
0 vs. 2	−13.80	<0.001
0 vs. 3	−23.90	<0.001
0 vs. 4	−8.05	0.001
1 vs. 2	−13.64	<0.001
2 vs. 3	−11.16	<0.001
2 vs. 4	−0.64	0.55
3 vs. 4	6.965	0.002

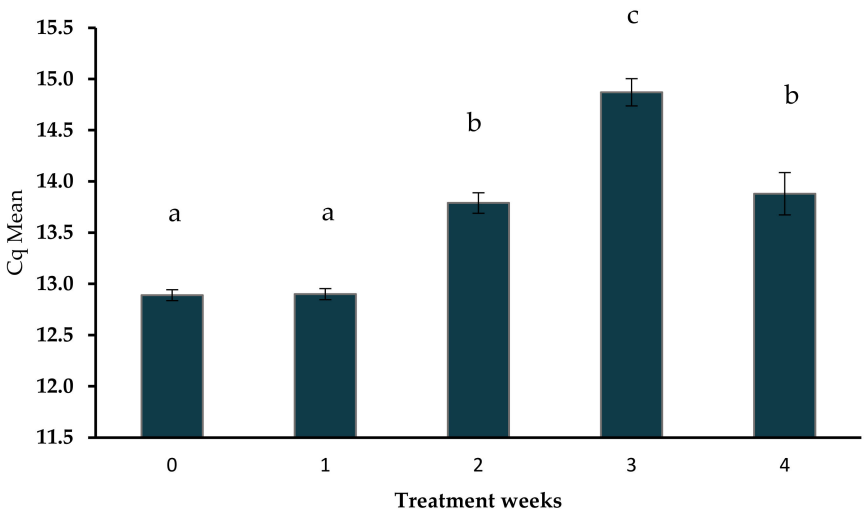

Figure 3. Effect of the treatment weeks over the Cq mean; different letters mean significant differences ($p < 0.05$) in the *t*-test.

When studying the copies present in the different samples of the mcrA gene, a reduction in the methanogen populations was observed, with a minimum of 36.57% in week 2 to a maximum of 63.01% in week 3. Similarly, in week 4, growth in the methanogen population was observed, but it was still 39.10% lower than the population detected before applying the biological activator (Table 5).

Table 5. Reduction in the mcrA gene copies and the treatments. All the comparisons are made with treatment week 0 and expressed in % of reduction.

Treatment Weeks	mcrA Copy Number in 50 µL	Reduction in Copies with Treatment (%)
0	7.11×10^7	
1	7.09×10^7	0.28
2	4.51×10^7	36.57
3	2.63×10^7	63.01
4	4.33×10^7	39.10

The ability of syncoms to control pathogens in agriculture has been widely documented [57–62], for example, organic matter production or foul odors reduction to control diseases and pests. Although inputs based on a single organism have been adequate for the control of various pathogens and pests, in many cases, the use of a mixture of microorganisms is more effective due to the interaction of these synthetic communities with the microbiota present in the soil [63,64]. One of the most extensive applications at the agricultural level of the use of mixtures of microorganisms is the reduction or elimination of foul odors during composting processes [25]. In this case, fungi and bacteria serve as inoculum to increase the rate of degradation of organic matter or to replace populations that cause anaerobic decomposition processes [23,65].

Products of the movement of microorganisms achieve this displacement or change in the structure of the community including syncoms. It has been documented that the application of these syncoms can be used for the control of human pathogens, for example, by displacing the populations of *Salmonella* spp., *Listeria* spp. and *Escherichia coli* through the production of acids, alcohols, and secondary metabolites of lactobacilli and yeasts [66]. Similarly, yeasts affect methanogenesis, reducing populations of methanogen bacteria and stimulating acetogenesis [67–70]. For their part, lactic acid bacteria also reduce methane production [71] due to the fact of lactic acid production. The application of the bio-activator in the different slurry samples made it possible to measure the reduction in the population of methanogen bacteria, probably due to the effect of the populations included in the input. This reduction was reflected in the decrease in methane emissions.

3.3. Emission Concentrations at the Barns

The measurements and sampling in this study were designed to have the most similar conditions in terms of the number of animals, bedding materials, and floor type. Nonetheless, the management and amount of manure, as well as the farm's architectural volume, differed in each barn. Because each had a different form and structure, the results showed that the architectural volume influenced how the air circulated in the barn. Figure 4B, for example, depicts each barn's passive air entrance. Overall, F2 and F3 had limited access to air entering through windows or doors, with four different air entrances of passive air on the farm; F6 had the most for the study. The results show how the emissions concentrations differed among the farms. Gas concentrations in some areas were significantly higher than in others, depending on the influence of the passive air entrance, particularly in its corners or where no direct passive airflow was present. For example, F2 and F3 had similar architectural volumes, with only one side of the air entrance, resulting in the highest emissions in some spots among all barns measured. The lowest emissions, on the other hand, were observed in F6, where the air entrance included all three sides and one door entrance. As a result, the architectural volume influenced how emissions were concentrated while excretes remained in the barns, with the highest emission points among the farms located in corners with no direct air access to the barn. This resulted in architectural volume recommendations for future barn designs in order to avoid the concentration of these gases inside the barn's facilities (Figure 4).

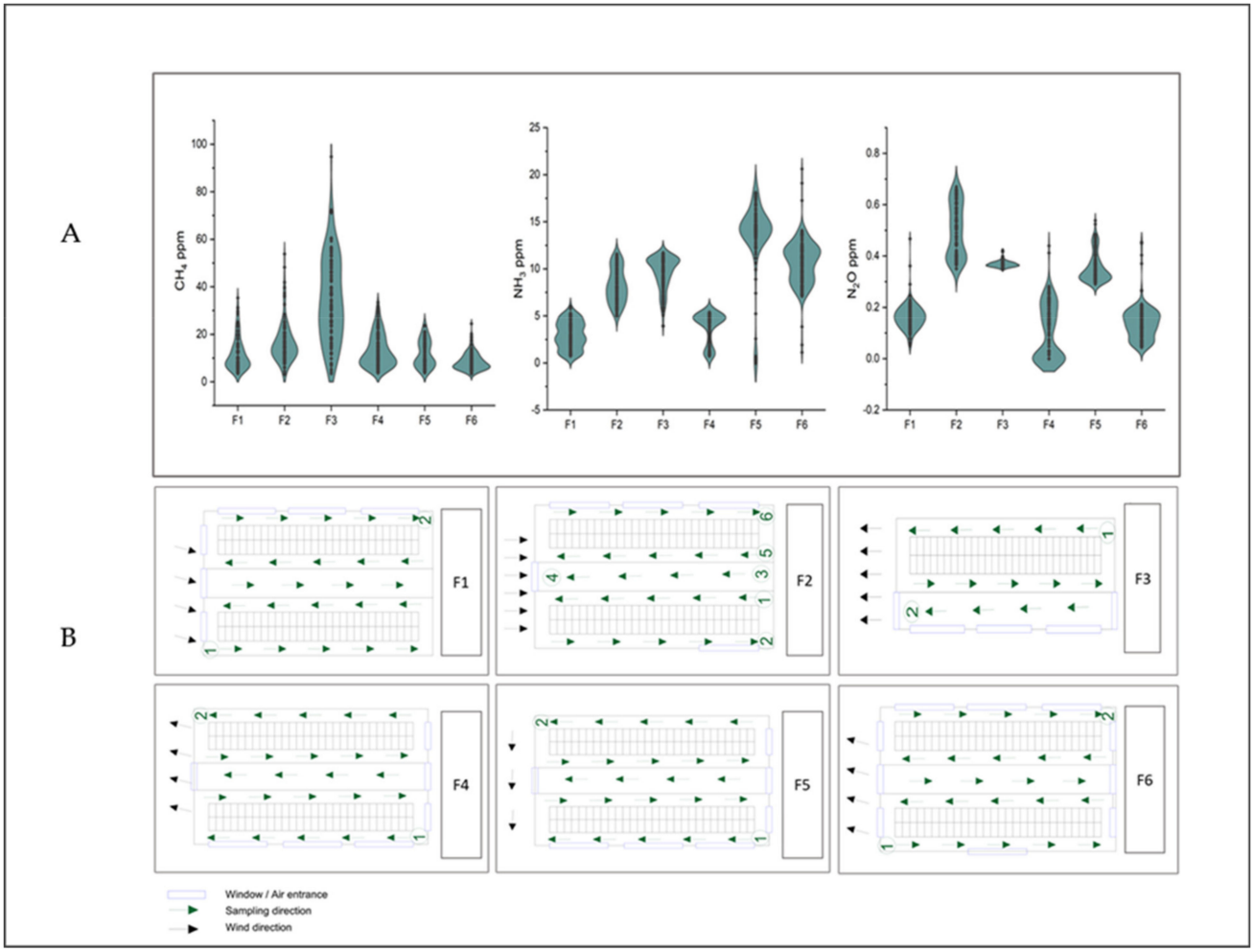

Figure 4. (**A**) Emission concentrations of CH_4, N_2O, and NH_3 in all the barns; (**B**) diagram of the measurement sampling and architectural volume in the barns.

In different studies, CH_4, N_2O, and NH_3 emissions increased with increasing temperature, leading to one of the possible differences among the barns shown in Figure 4A. Animal excreted fecal matter undergoes a series of chemical reactions. These include decomposition, hydrolysis, nitrification, denitrification, and fermentation, among others, from which N_2O can be produced [72]. However, the main trigger of its formation is the temperature in the manure [71].

More than analyzing the source of emissions, the aim was to establish a certain level among the farms in order to understand how emissions fluctuate and to obtain similarities or differences in concentrations in each farm using a commercial product. When excrements were on the floor, the commercial product was usually sprayed among the farms on a daily basis. However, the findings suggest that the commercial product may not have an immediate impact on emission reduction after being applied to the barns. One explanation is that syncoms require a specific time for a growing population and begin the displacement to compete with the methanogen populations after application. As a result, the time that the excretes remain on the floor with the commercial product is limited (no more than 3 h) in order to increase the growing population of syncoms after the application. Furthermore, bio-activators are recommended as soon as the excreta are on the ground. Adding the product to the floor promotes further activation and reproduction of the communities during slurry storage and reduces emissions during slurry storage. Additional factors are recommended for future evaluations and modeling of the emissions. In addition to the slurry management and architectural volume, these factors are the slurry temperature, the quantity of the slurry inside the barn, and cattle breathing.

4. Conclusions

The present study provided a comprehensive summary of GHG emissions reductions for dairy cattle slurry treatment utilizing a bio-activator. For CH_4 and N_2O emissions, GHG reductions of at least 50% were achieved. Syncoms have been shown to reduce methanogen populations and emissions during slurry storage, correlating with the profile of methane reduction during the performance of the slurry measurements among the farms. This is consistent with the 36–63 percent decline in methanogenic populations found after CH_4 removal. In addition, farm management and architectural volume had a significant impact on CH_4, N_2O, and NH_3 emission concentrations. More research is necessary to observe how the slurry with syncom microorganisms interacts in the soil and how it can improve nitrogen fixation and carbon storage in grassland and other areas where the slurry is applied. This is an option that could help mitigate the effects of climate change. On the other hand, it is critical that the farms are managed properly, in accordance with the activation and operating recommendations for bio-activator products, as well as adequate slurry management. As there is scant information regarding the benefits and use of bio-activators in intensive livestock production in Spain, this research is significant for understanding and observing their performance. In addition, this research can provide data for future assessments and climate mitigation options in agricultural practices.

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