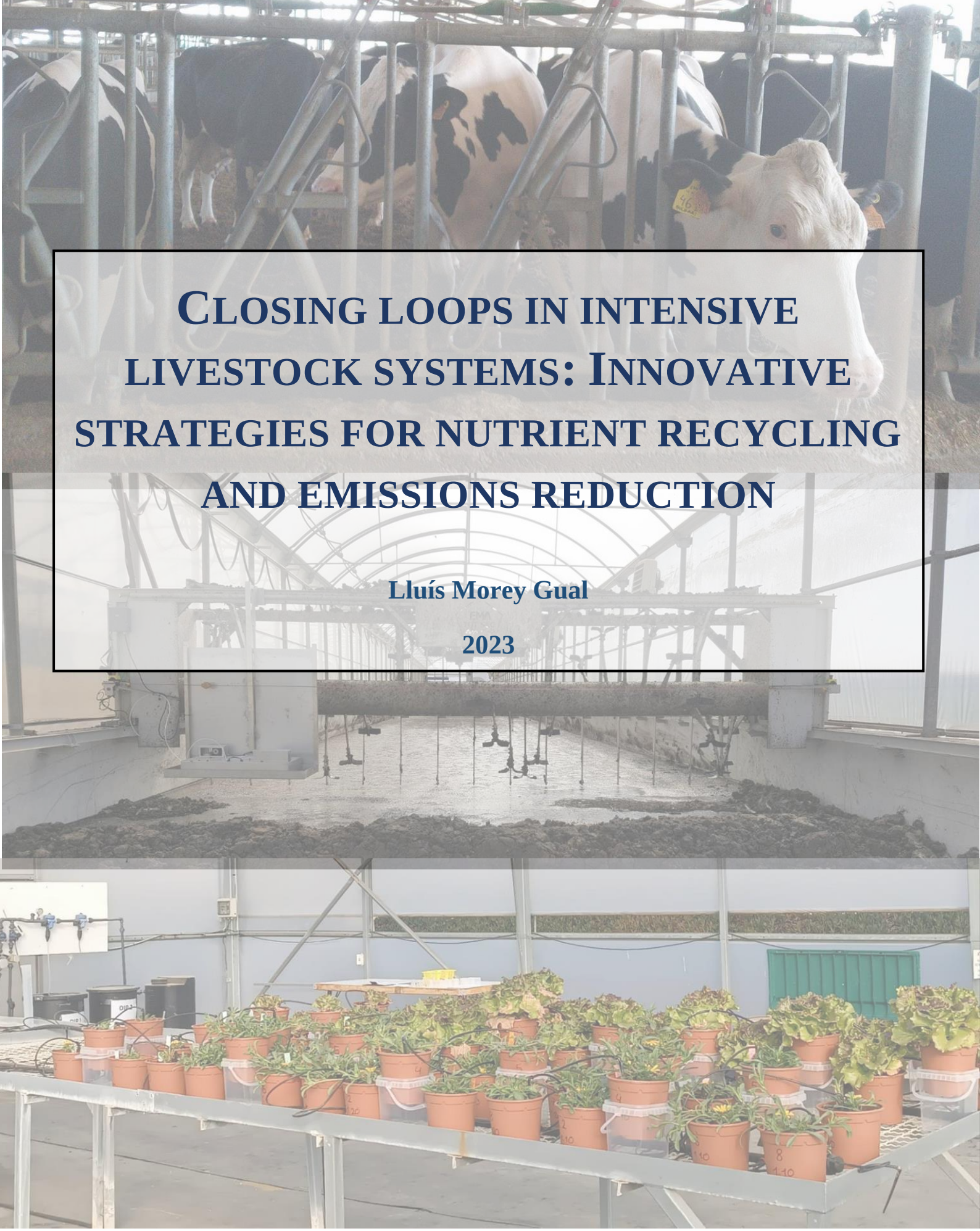


CLOSING LOOPS IN INTENSIVE LIVESTOCK SYSTEMS: INNOVATIVE STRATEGIES FOR NUTRIENT RECYCLING AND EMISSIONS REDUCTION

Lluís Morey Gual

2023

UNIVERSITAT POLITÈCNICA DE CATALUNYA BARCELONATECH

IRTA Institute of Agrifood Research and Technology

UNIVERSITEIT GENT

Catalan translation of the title

Tancant els cicles en sistemes ramaders intensius: estratègies innovadores per al reciclatge de nutrients i la reducció d'emissions

Dutch translation of the title

Het sluiten van kringlopen in intensieve veehouderijsystemen: innovatieve strategieën voor nutriëntenrecycling en emissiereductie

Spanish translation of the title

Cerrando ciclos en sistemas ganaderos intensivos: estrategias innovadoras para el reciclaje de nutrientes y la reducción de emisiones

Funding

This doctoral thesis has been performed and financed in the framework of the Horizon 2020 projects "Circular Agronomics" (GA 773649; https://www.circularagronomics.eu/) and "Nutry2Cycle" (GA 773682; https://www.nutri2cycle.eu/). The author thanks the financial support of AGAUR (Scholarship number 2019FI_B00694), from the Generalitat de Catalunya, and the support of the CERCA Program and the Consolidated Research Group TERRA (ref.2017SGR1292), both from the Generalitat de Catalunya.

PhD program in Environmental Engineering by Universitat Politècnica de Catalunya

PhD program in Bioscience Engineering by Ghent University

Doctoral thesis by:

Lluís Morey Gual

Thesis supervisors:

Víctor Riau Arenas (IRTA), Marta Terré Trullà (IRTA), Erik Meers (UGENT)

Thesis advisor:

Santiago Gassó Domingo (UPC)

IRTA-Sustainability in Biosystems & Ruminant Production programs

UPC-Department of Civil and Environmental Engineering

UGENT-Department of Green Chemistry and Technology

Barcelona, June 2023

Abstract

The exponential growth of world population is leading to a high demand of food products and, consequently, to the development of intensive agricultural and livestock systems. In this sense, synthetic fertilizers are being used in a non-sustainable way to produce food and feedstock, requiring massive amounts of energy and depleting mineral resources, with a volatile fluctuation of their prices due to geopolitical conflicts. At the same time, intensive livestock production is still inefficient in terms of nutrient use and contributes to a half of the amount of greenhouse gases and most of ammonia emissions of agriculture.

In this context, the recovery of biobased fertilizers and bioenergy from animal manure to partially replace synthetic mineral fertilizers and fossil fuels, as well as strategies to reduce emissions along the manure management chain, should be considered as a key approach to move towards a more sustainable and resilient agriculture and livestock production. This doctoral thesis has looked to contribute to this aim by developing different innovative strategies at two main levels:

(i) **At animal production level**, precision feeding tools have been used to adjust diets in dairy cows based on individual animal requirements, reducing the amount of nitrogen present on the excreta, and the potential volatilization of ammonia. Nitrogen was reduced in urine and manure by 28% and 19% respectively without affecting milk production, and a reduction trend on ammonia emission of 20% during manure storage was observed.

Besides, samples of dairy cow ruminal fluid were obtained to isolate and identify hyper ammonia-producing bacteria that highly contributes to ammonia production in cows' metabolism. After the identification of *Clostridium sporogens*, *Terrisporobacter glycolicus* (*Clostridium glycolicum*), *Megasphaera elsdenii*, *Clostridium argentinense, Streptococcus sp., Prevotella ruminicola, and Acidaminococcus fermentans*, an *in vitro* study using bacteriophages isolated from the same ruminal fluid were inoculated as a population reduction strategy. Unfortunately, none of the bacteriophages present in the rumen was able to interact with the bacteria.

(ii) **At manure treatment and valorization level**, agro-industrial anaerobic digestate, the solid fraction of the digestate, and a mixture of the solid fraction with a low nitrogen stream from a stripping/scrubbing unit were dried in a solar drying greenhouse system, in some cases combined with acidification with sulfuric acid, to produce organic NPK fertilizers, while reducing ammonia and greenhouse gas emissions during the process. All dried products, both acidified and non-acidified, meet the current European Regulation for solid NPK fertilizers with some exceptions due to the zinc content. Moreover, acidification proved to reduce up to 94% ammonia emissions during the process when comparing the acidified to the non-acidified products. In addition, the resulting products were tested for phytotoxicity with germination and plant pot trials with lettuce. Except for the acidified dried digestate, the rest of the products did not produce any toxicity effect on germination, and the dried acidified digestate showed a biostimulator effect (Germination

index > 120%). During the pot trials, the fertilizers showed an efficient performance, always lower than the synthetic fertilizer, but similar in the case of the acidified products, and better than the negative control. All of them fit the current regulations on heavy metals and potential toxic elements in soil and edible parts of the plant.

This doctoral thesis has developed suitable innovative strategies to close nutrient cycles and to move towards a more sustainable and resilient agri-food sector, by reducing nitrogen excretion by animals, recovering and reusing nutrients from manure and minimizing pollutant emissions in intensive livestock systems.

Resum

El creixement exponencial de la població mundial està portant a una gran demanda de productes alimentaris i, en conseqüència, al desenvolupament de sistemes agrícoles i ramaders intensius. En aquest sentit, els fertilitzants sintètics s'estan utilitzant de manera no sostenible per produir aliments i matèries primeres, que requereixen quantitats massives d'energia i esgoten els recursos minerals, amb una fluctuació volàtil dels seus preus per conflictes geopolítics. Al mateix temps, la ramaderia intensiva és encara ineficient pel que fa a l'ús de nutrients i contribueix a la meitat de la quantitat de gasos d'efecte hivernacle i a la majoria de les emissions d'amoníac de l'agricultura.

En aquest context, la recuperació de fertilitzants biològics i de bioenergia a partir de dejeccions animals per substituir parcialment els fertilitzants minerals sintètics i els combustibles fòssils, així com les estratègies per reduir les emissions al llarg de la cadena de gestió de les dejeccions, s'haurien de considerar com un enfocament clau per avançar cap a un sistema més sostenible, i una agricultura i una producció ramadera resilients. Aquesta tesi doctoral ha volgut contribuir a aquest objectiu desenvolupant diferents estratègies innovadores a dos nivells principals:

(i) **A nivell de producció animal**, s'han utilitzat eines d'alimentació de precisió per ajustar les dietes de les vaques lleteres en funció de les necessitats individuals dels animals, reduint la quantitat de nitrogen present a les excretes i la possible volatilització de l'amoníac. El nitrogen es va reduir en l'orina i les dejeccions en un 28% i un 19%, respectivament, sense afectar la producció de llet, i es va observar una tendència de reducció de les emissions d'amoníac del 20% durant l'emmagatzematge de les dejeccions.

A més, es van obtenir mostres de líquid ruminal de vaques lleteres per aïllar i identificar bacteris híper-productors d'amoníac que contribueixen substancialment a la producció d'amoníac en el metabolisme de les vaques. Després de la identificació de *Clostridium sporogens*, *Terrisporobacter glycolicus (Clostridium glycolicum)*, *Megasphaera elsdenii*, *Clostridium argentinense*, *Streptococcus sp.*, *Prevotella ruminicola* i *Acidaminococcus fermentans*, es va fer un estudi *in vitro* en el que es va inocular bacteriòfags del mateix líquid ruminal com a estratègia de reducció de població. Malauradament, cap dels bacteriòfags presents al nostre rumen va poder interactuar amb els bacteris.

(ii) A nivell de tractament i valorització de les dejeccions, es van assecar digestat agroindustrial, la fracció sòlida del digestat i una barreja de la fracció sòlida amb una corrent baixa en nitrogen procedent d'una unitat de stripping en un sistema d'assecat solar, en alguns casos combinats amb acidificació amb àcid sulfúric, per produir fertilitzants orgànics NPK, alhora que es reduïen les emissions d'amoníac i gasos d'efecte hivernacle durant el procés. Tots els productes secs, tant acidificats com no acidificats, compliren amb el Reglament europeu vigent per a fertilitzants sòlids NPK amb algunes excepcions pel contingut de zinc. A més, l'acidificació

va demostrar reduir fins a un 94% les emissions d'amoníac durant el procés en comparar els productes acidificats amb els no acidificats. Els productes resultants van ser provats amb un experiment de germinació i assajos en test amb enciam per determinar la seva fitotoxicitat. Excepte el digestat sec acidificat, la resta de productes no van produir cap efecte de toxicitat en la germinació, i el digestat sec acidificat va mostrar un efecte bioestimulador (índex de germinació > 120%). Durant les proves en test, els fertilitzants van mostrar un rendiment eficient, sempre inferior al sintètic, però similar en el cas dels productes acidificats, i millor que el control negatiu. Tots ells s'ajustaren a la normativa vigent sobre metalls pesants i elements potencialment tòxics al sòl i a les parts comestibles de la planta.

Aquesta tesi doctoral ha desenvolupat estratègies innovadores adequades per tancar els cicles de nutrients i avançar cap a un sector agroalimentari més sostenible i resilient, mitjançant la reducció de l'excreció de nitrogen per part dels animals, la recuperació i reutilització de nutrients de les dejeccions i la minimització de les emissions contaminants en els sistemes ramaders intensius.

Samenvatting

De exponentiële groei van de wereldbevolking leidt tot een grote vraag naar voedselproducten en bijgevolg tot de ontwikkeling van intensieve landbouw- en veeteeltsystemen. In deze zin worden synthetische meststoffen op een niet-duurzame manier gebruikt om voedsel en grondstoffen te produceren, waarbij enorme hoeveelheden energie nodig zijn en minerale bronnen uitgeput raken, met een volatiele schommeling van hun prijzen als gevolg van geopolitieke conflicten. Tegelijkertijd is intensieve veeteelt nog steeds inefficiënt wat betreft het gebruik van voedingsstoffen en draagt het bij aan de helft van de hoeveelheid broeikasgassen en het grootste deel van de ammoniakuitstoot van de landbouw.

In deze context moet de terugwinning van biogebaseerde meststoffen en bio-energie uit dierlijke mest ter gedeeltelijke vervanging van synthetische minerale meststoffen en fossiele brandstoffen, evenals strategieën om de emissies in de mestbeheerketen te verminderen, worden beschouwd als een belangrijke benadering om te komen tot een duurzamere en veerkrachtigere landbouw en veeteelt. Deze doctoraatsthesis heeft getracht bij te dragen tot dit doel door verschillende innovatieve strategieën te ontwikkelen op twee belangrijke niveaus:

(i) Op het niveau van de dierlijke productie werden precisievoederinstrumenten gebruikt om het rantsoen van melkkoeien aan te passen op basis van de individuele behoeften van het dier, waardoor de stikstofefficiëntie werd verbeterd en de hoeveelheid stikstof in de uitwerpselen en de potentiële vervluchtiging van ammoniak werden verminderd. Stikstof in urine en mest werd verminderd met respectievelijk 28% en 19% zonder de melkproductie te beïnvloeden, en er werd een reductietrend van 20% op ammoniakemissie tijdens mestopslag waargenomen.

Daarnaast werden monsters van pensvloeistof van melkkoeien verkregen om hyperammoniakproducerende bacteriën te isoleren en te identificeren die in hoge mate bijdragen aan de ammoniakproductie in het metabolisme van koeien. Na de identificatie van *Clostridium sporogens, Terrisporobacter glycolicus (Clostridium glycolicum), Megasphaera elsdenii, Clostridium argentinense, Streptococcus sp., Prevotella ruminicola* en *Acidaminococcus fermentans,* werd een in vitro studie uitgevoerd met bacteriofagen geïsoleerd uit dezelfde pensvloeistof als een strategie om de populatie te reduceren. Helaas was geen van de bacteriofagen in de pens in staat tot interactie met de bacteriën.

(ii) Op het niveau van mestverwerking en -valorisatie werden agro-industrieel anaerobe digestaat, de vaste fractie van het digestaat en een mengsel van de vaste fractie met een stikstofarme stroom uit een strip-/schrobinstallatie gedroogd in een kasinstallatie voor droging op zonne-energie, in sommige gevallen gecombineerd met aanzuring met zwavelzuur, om organische NPK-meststoffen te produceren, terwijl de ammoniak- en broeikasgasemissies tijdens het proces werden verminderd. Alle gedroogde producten, zowel aangezuurde als niet-aangezuurde, voldoen aan de huidige Europese regelgeving voor vaste NPK-meststoffen, met

enkele uitzonderingen vanwege het zinkgehalte. Bovendien bleek de aanzuring de ammoniakemissies tijdens het proces tot 94% te verminderen wanneer de aangezuurde met de niet-aangezuurde producten worden vergeleken. Bovendien werden de resulterende producten getest op fytotoxiciteit door middel van kiem- en potproeven met sla. Met uitzondering van het aangezuurde gedroogde digestaat, hadden de rest van de producten geen toxisch effect op de kieming, en het gedroogde aangezuurde digestaat vertoonde een biostimulerend effect (kiemindex > 120%). Tijdens de potproeven toonden de meststoffen een efficiënte prestatie, altijd lager dan de kunstmest, maar vergelijkbaar in het geval van de aangezuurde producten, en beter dan de negatieve controle. Ze voldeden allemaal aan de huidige regelgeving voor zware metalen en potentieel giftige elementen in de bodem en eetbare delen van de plant.

In dit proefschrift zijn geschikte innovatieve strategieën ontwikkeld om nutriëntenkringlopen te sluiten en te komen tot een duurzamere en veerkrachtigere agrovoedingssector, door de stikstofuitscheiding door dieren te verminderen, nutriënten uit mest terug te winnen en te hergebruiken en de uitstoot van verontreinigende stoffen in intensieve veeteeltsystemen te minimaliseren.

Resumen

El crecimiento exponencial de la población mundial está potenciando una gran demanda de productos alimenticios y, en consecuencia, al desarrollo de sistemas agrícolas y ganaderos intensivos. En este sentido, los fertilizantes sintéticos se están utilizando de forma no sostenible para producir alimentos y materias primas, que requieren cantidades masivas de energía y agotan los recursos minerales, con una fluctuación volátil de sus precios por conflictos geopolíticos. Al mismo tiempo, la ganadería intensiva es todavía ineficiente en lo que se refiere al uso de nutrientes y contribuye a la mitad de la cantidad de gases de efecto invernadero ya la mayoría de las emisiones de amoníaco de la agricultura.

En este contexto, la recuperación de fertilizantes biológicos y de bioenergía a partir de estiércoles animales para sustituir parcialmente los fertilizantes minerales sintéticos y los combustibles fósiles, así como las estrategias para reducir las emisiones a lo largo de la cadena de gestión de estiércol, se deberían considerar como un enfoque clave para avanzar hacia un sistema más sostenible y una agricultura y una producción ganadera resilientes. Esta tesis doctoral ha querido contribuir a este objetivo desarrollando diferentes estrategias innovadoras a dos niveles principales:

(i) A nivel de producción animal, se han utilizado herramientas de alimentación de precisión para ajustar las dietas de las vacas lecheras en función de las necesidades individuales de los animales, reduciendo la cantidad de nitrógeno presente en las excretas y la posible volatilización del amoníaco. El nitrógeno se redujo en la orina y en las heces en un 28% y un 19%, respectivamente, sin afectar a la producción de leche, y se observó una tendencia de reducción de las emisiones de amoníaco del 20% durante el almacenamiento del estiércol.

Además, se obtuvieron muestras de con líquido ruminal de vacas lecheras para aislar e identificar bacterias híper-productoras de amoníaco que contribuyen sustancialmente a la producción de amoníaco en el metabolismo de las vacas. Tras la identificación de *Clostridium sporogens, Terrisporobacter glycolicus (Clostridium glycolicum), Megasphaera elsdenii, Clostridium argentinense, Streptococcus sp., Prevotella ruminicola* y *Acidaminococcus fermentans*, se realizó un estudio *in vitro* en el que se inocularon bacteriófagos del mismo líquido ruminal. Desgraciadamente, ninguno de los bacteriófagos presentes en nuestro rumen pudo interactuar con las bacterias.

(ii) A nivel de tratamiento y valorización de estiércol, se secaron digestato agroindustrial, la fracción sólida del digestato y una mezcla de la fracción sólida con una corriente baja en nitrógeno procedente de una unidad de stripping en un sistema de secado solar, en algunos casos combinados con acidificación con ácido sulfúrico, para producir fertilizantes orgánicos NPK, al tiempo que se reducían las emisiones de amoníaco y gases de efecto invernadero durante el proceso. Todos los productos secos, tanto acidificados como no acidificados, cumplieron con el

Reglamento europeo vigente para fertilizantes sólidos NPK con algunas excepciones por el contenido de zinc. Además, la acidificación demostró reducir hasta en un 94% las emisiones de amoníaco durante el proceso al comparar los productos acidificados con los no acidificados. Los productos resultantes fueron probados con un experimento de germinación y ensayos en maceta con lechuga para determinar su fitotoxicidad. Excepto el digestato seco acidificado, el resto de los productos no produjeron ningún efecto de toxicidad en la germinación y el t seco acidificado mostró un efecto bioestimulador (índice de germinación > 120%). Durante las pruebas en test, los fertilizantes mostraron un rendimiento eficiente, siempre inferior al sintético pero similar en el caso de los productos acidificados, y mejor que el control negativo. Todos ellos se ajustaron a la normativa vigente sobre metales pesados y elementos potencialmente tóxicos en el suelo y en las partes comestibles de la planta.

Esta tesis doctoral ha desarrollado estrategias innovadoras adecuadas para cerrar los ciclos de nutrientes y avanzar hacia un sector agroalimentario más sostenible y resiliente, mediante la reducción de la excreción de nitrógeno por parte de los animales, la recuperación y reutilización de nutrientes del estiércol y la minimización de las emisiones contaminantes en los sistemas ganaderos intensivos.

Acknowledgments

I would like to thank all the people involved somehow in the development of this thesis. Plant operators, administration teams, sampling campaigns and laboratory co-workers and supervisors.

First of all, I want to acknowledge the entire GIRO (nowadays Sustainability and Biosystems) team for making me feel part of their family since 2013. It has been a long journey full of friendships, training and learning, sampling adventures, and a lot of laughs and fun. All thanks to the inestimable introduction to this world by one of the best professors I ever had: Xavier Flotats i Ripoll.

I cannot imagine this path without my family, who since I was born helped me to become the (all mighty) person I am today, or without my friends, who even though they thought I was using manure to produce rocket fuel to go on a spatial mission to the moon, they have been at my side every day. Thanks to all of them for making this rocky road to the end of the Ph.D. more tolerable.

Moreover, I would like to thank the group of molecular microbiology from UAB and Elena Garcia from IRTA for their help and support during the development of the microbiological part of the thesis.

I cannot forget either of the Re-Source Lab at Ghent University, led by Erik Meers (co-supervisor of the thesis), where I spent the last part of the Ph.D. surrounded by fantastic people, professional and friendly, in the beautiful city of Ghent.

More individually, I need to thank the huge effort done by my IRTA supervisors Víctor and Marta, and not supervisor in paper but in work, Belén. You all pushed me through this, helping me in the most complicated moments.

And thanks to you Marcella, for enlightening my dark days and stay by me side making me feel capable of anything.

Table of contents

List of Tables

List of Figures

Acronyms and Abbreviations

AD	AD
ADF	Acid Detergent Fiber
AIA	Acid Insoluble Ash
AMS	Automatic Milking System
Annamox	Anaerobic ammonium oxidation
aNDF	NDF assayed with a heat stable amylase and expressed exclusive of residual ash
ASF	Acidified Solid Fraction of Digestate
BW	Body Weight
C	Carbon
Ca	Calcium
CAP	Common Agricultural Policy
Cd	Cadmium
CE	Conformité Européenne
CEAP	Circular Economy Action Plan
CH_4	Methane
CHP	Combustion Heat Power
Co	Cobalt
CO_2	Carbon dioxide
CONV	Conventional feeding
CP	Crude Protein
Cr (VI)	Cromium VI
Cu	Copper
DAD	Dried Acidified Digestate
DASF	Dried Acidified Solid Fraction of Digestate
DD	Dried Digestate
DIM	Days In Milk
DLA	Double Layer Agar
DM	Dried Mixture
DW	Dry Weight
DNA	Deoxyribonucleic Acid
DSF	Dried Solid Fraction of Digestate
EC	Electrical Conductivity
ECHA	European Chemicals Agency
ECM	Energy Corrected Milk
ENU	Efficiency of Nitrogen Utilization
EU	European Union

EVAM	Estació de Vacum de Monells
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
Fe	Iron
FPR	Fertilizer Product Regulation
GHG	Greenhouse Gases
HAP	Hyper-ammonia producing bacteria
Hg	Mercurium
K	Potassium
K_2O	Potassium oxide
KCl	Potassium chloride
kWh	Kilowatt-hour
LF	Liquid Fraction of Digestate
LN	Lactation Number
Lys	Lysine
Met	Methionine
Mg	Magnesium
MJ	Mega Joules
Mn	Manganese
MUN	Milk Urea Nitrogen
N	Nitrogen
N_2O	Nitrous oxide
Na	Sodium
NDF	Neutral Detergent Fiber
NE	Net Energy
NECD	National Emission Ceilings Directive
NEL	Net Energy for Lactation
NFC	Non Fiber Carbohydrates
NH_3	Ammonia
NH_4^+	Ammonium
Ni	Nikel
NIH	National Institute of Health
NO_2	Nitrogen Dioxide
NO_3^-	Nitrate
NPK	Nitrogen-Phosphorus-Potassium
NPN	Non Protein Nitrogen
OC	Organic Carbon

OM	Organic Matter
P	Phosphorus
P_2O_5	Phosphorus pentoxide
Pb	Lead
PMR	Partial Mixed Ration
PREC	Precision feeding
PTE	Potential Toxic Elements
rDNA	recombinnt Deoxyribonicleic Acid
RDP	Rumen Degradable Protein
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
RUP	Rumen Undegradable Protein
S	Sulfur
SAFA	Sustainability Assessments of Food and Agriculture systems
SCC	Somatic Cell Count
SD	Standard Deviation
SF	Solid Fraction of Digestate
SLF	Stripped Liquid Fraction low in N
SPAD	Relative leaf chlorophyll content
TAN	Total Ammonia Nitrogen
TC	Total Carbon
TEM	Transmision Electron Microscopy
TK	Total Potassium
TMR	Total Mixed Rations
TN	Total Nitrogen
TOC	Total Organic Carbon
TP	Total Phosphorus
TS	Total Solids
UASB	Upflow Anaerobic Sludge Blanket
VS	Voltile Solids
Zn	Zinc

Chapter 1 – Introduction

Population growth has exponentially increased in the last century, with the world population rising from 3.7 billion inhabitants in 1970 to 7.9 billion in 2021, and a forecast of reaching 9.7 billion by 2050 (Koul et al., 2022; United Nations 2022; Walling and Vaneeckhaute, 2020). This has led to a drastic increase in food consumption and, consequently, the need of a higher and more intensive livestock and agricultural production. Agricultural resources demand is estimated to become 15% higher in the next decade and 50-100% higher by 2050, with the associated increase in waste generation, greenhouse gases (GHG) and ammonia (NH_3) emissions, and depletion of phosphorus (P) and potassium (K) minerals, while increasing energy consumption derived from the production of nitrogen-based chemical fertilizers (Li and Cai, 2022; Walling and Vanneckhaute, 2020; Tripathi *et al*., 2019; OECD, 2018).

In this regard, from 1960 to 2019, mineral fertilizers production from non-renewable and high-energy demanding sources increased by 366% for potassium fertilizers, 285% for phosphorous fertilizers, and 870% for nitrogen fertilizers (FAOSTAT, 2022) (Figure 1), growing from 50 million tons to 200 million tons of worldwide total fertilizer consumption (Ritchie *et al*., 2022). In 2020, the amount of mineral fertilizer used in the European Union's agricultural sector was 11.2 million tons (10 million tons of mineral nitrogen (N) and 1.2 million tons of mineral P, Eurostat, 2022), with more than 45% of the total N applied coming from mineral fertilizers (Hendriks *et al*., 2022).

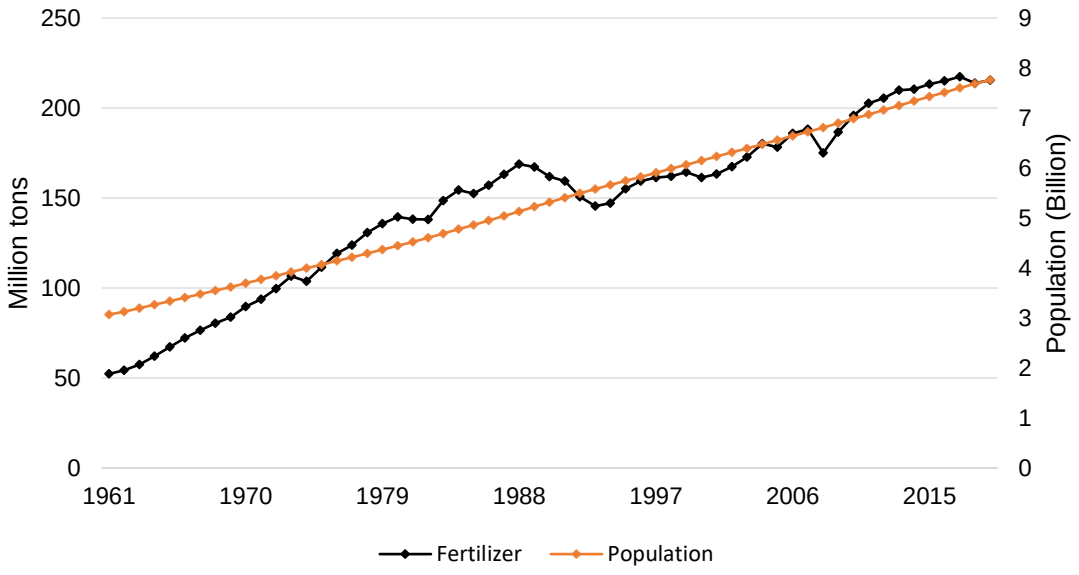


Figure 1. World total fertilizer consumption and population growth from 1961 to 2019 including nitrogen, phosphorus, and potassium. Data from: United Nation, World Population Prospects 2022; USDA 2022.

Concerning the depletion of natural resources for manufacturing NPK mineral fertilizers, and its associated impacts, most of the N-based mineral fertilizer is produced throughout the Haber-

Bosch process, one of the most energy-consuming industrial processes, representing 1-2% of global energy consumption (Kyriakou 2020), mainly from natural gas, and 1-2% of the anthropogenic CO_2 emissions (Yüzbaşıoğlu *et al.*, 2022). On the other hand, the main P source to produce phosphorous fertilizers is the phosphate rock, which has long regeneration times, causing interruptions in the P-rock distribution. In fact, it is foreseen that phosphorous rock resources will be depleted during the 21st century (Arrobas *et al*., 2022). Since phosphate extraction is carried out outside of the European Union (EU), P mining also leads to high production and transportation costs (Arcas-Pilz *et al*., 2022). The availability of K-based fertilizers, such as KCl, is limited for developing countries since their global production takes place in only five countries: Canada, Belarus, Russia, China and Germany (Swoboda *et al*., 2022). Likewise, the industry of N-based fertilizers in Europe depends to a large extent on gas coming from Russia and, likewise, Russia and Belarus play a key role in the world production of P rock-based fertilizers. The prices of mineral fertilizers increased up to 5 times since the beginning of this century, from 100-200€/ton to 900-1000€/ton, and the current geopolitical situation is leading to a further increase of fertilizer prices, which has a direct impact on the use of non-renewable fertilizers in agriculture in the EU (Eurostat, 2022; FASUSDA, 2022).

From the point of view of the performance of chemical fertilizers in soil and crop production, recent works have demonstrated that the nutrient uptake efficiency from chemical fertilizers in crops is around 30-50%, while the rest of nutrients are lost through microorganism degradation, hydrolysis, leaching, and photodegradation (Li and Cai, 2022; Mózner *et al*., 2012). Moreover, the use of chemical fertilizers is also associated with environmental problems such as soil degradation and compaction, water bodies contamination through leaching, and greenhouse gas emissions (Li and Cai, 2022). All these concerns make it necessary to find more sustainable processes for the production of fertilizers, optimizing the use of energy and resources, and reducing the pollution of the production processes as well as soil-water contamination.

To support sustainable agriculture and accomplish the objective of the European Union to manage 25% of the land organically by 2030, organic fertilizers and biobased fertilizers have emerged as a promising solution to reduce the high inputs of chemical fertilizers, included in the European projections and plans (CEAP, 2020; EC, 2019; EC, 2020). The European Commission introduced the European Green Deal in December 2019 as a sustainable growth agenda for the upcoming years focusing on obtaining no net emissions of GHG by 2050 and on decoupling economic growth from resource use. This Green Deal includes the Circular Economy Action Plan and the Farm to Fork Strategy - the first focuses on reducing pressure on natural resources by promoting initiatives to the entire life cycle of products while the latter works towards making food systems fair, healthy, and environmentally friendly by reducing by 50% the use of chemical and hazardous pesticide by 2030.

Livestock manure, compost, organic waste from the food processing industry, municipal solid waste and agricultural waste have the potential to become organic fertilizers (Bergstrant *et al*., 2022; Fang *et al.*, 2021). In comparison with chemical or synthetic fertilizers, organic fertilizers improve soil porosity promoting nutrient balance. Moreover, they are a source of vitamins, hormones, macro and micronutrients, and enzymes that can have an important function in crop quality and soil fertility (Rasouli *et al*., 2022). Despite being a promising solution and even traditionally used (e.g., livestock manure) (Loss *et al*., 2019), the direct use or the required treatment technologies applied to these fertilizing products have some disadvantages such as the need for land treatment, nutrient losses, gas emissions, long operation time, and secondary pollution (Li and Cai, 2022).

Moreover, the livestock sector is responsible for almost a half of the GHG emissions (42%) of agriculture, mostly associated with enteric fermentation, manure management, storage and field application (Menardo *et al*., 2021). The most influential GHG emissions are nitrous oxide (N_2O), methane (CH_4), and carbon dioxide (CO_2) (Walling and Vaneeckhaute, 2020). Besides, NH_3 (considered an acidifying gas) represents the most important gas emitted by the livestock sector (about 80-90% of global NH_3 emissions) and plays an important role in GHG emissions as it can be transformed into N_2O by ammonia-oxidizing bacteria (Prosser *et al*., 2020; Uwizeye *et al*., 2020). For instance, when raw manure is directly applied to the soil as a fertilizer, more than 50% of the NH_3 can be released during the first day (Sigurnjack *et al*., 2017). This emitted NH_3 affects air quality and causes eutrophication, loss of biodiversity, and water and soil acidification when it is deposited in land or water bodies (Zhang *et al*., 2021).

Many agents can influence the production of GHG and NH_3 throughout the whole agronomic chain, such as the protein content and diet of livestock, the manure storage system used, and the fertilizer application method to the field (Menardo *et al*., 2021; Zhang *et al*., 2021). Therefore, to reduce the emissions release of animal husbandry, different works have modified animal diets and studied manure treatment and management systems. Zhang *et al*., (2019) suggested that acidification of manure can reduce the amount of volatilized NH_3, as the chemical equilibrium favors the ionized and non-volatile form (NH_4^+) of ammonia-ammonium. Zhang *et al*. (2021) proposed the use of different covers during manure storage to reduce NH_3 emissions. However, the use of one technology to control one component can affect the rest. For example, VanderZaag *et al*., (2009) found that the use of straw as a bedding material combined with dairy manure reduces NH_3 emissions but increases N_2O ones.

In this context, it is crucial to find efficient and innovative strategies to reduce the impact of the agricultural and livestock sectors on the environment in terms of N and carbon (C) losses via emissions and low nutrient uptake/use efficiency. The recovery of biobased fertilizers and bioenergy from animal manure to partially replace synthetic mineral fertilizers and fossil fuels,

as well as strategies at animal production level to reduce emissions along the manure management chain, should be considered as a key approach to move towards a more sustainable and resilient agriculture and livestock production. This doctoral thesis has looked to contribute to this aim by developing different innovative strategies within the livestock production chain both at Animal-farm and Manure valorization levels.

Animal-farm level

The livestock sector greatly contributes to the European Union's economy (170 billion euros), representing 40% of the total agricultural activity. In 2020, there were 113 million livestock units (Figure 2) being France, Spain and Germany the main producers; dairy and beef cattle represented 49%, followed by pig and poultry, with 30% and 15% respectively (Eurostat, 2023; Peyraud and MacLeod, 2020). The distribution in Catalonia, where this thesis was mainly developed, is quite different, led by far by poultry production. Nevertheless, manure production is higher than poultry manure; only in Catalonia, 9.4 million tons of pig and cow manure are generated per year compared to 2.8 million tons of poultry manure.

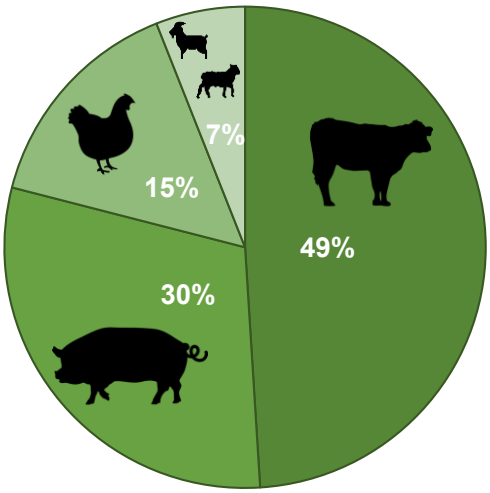


Figure 2. Livestock distribution in Europe 2020. Beef and dairy cattle are represented together. Data from Eurostat, (2023).

An increasing trend on milk production in Europe is foreseen for the following 10 years. It is estimated that milk production will reach 162 million tons, being the first world milk supplier with 30% of global trade (EC, 2021). In this sense, dairy farming is becoming a concern. CH_4, coming from enteric fermentation and manure management, contributes to almost a half of the total dairy farms' emissions (Gerber *et al.*, 2013), while the production of feedstock requires a large use of land and water consumption (Merril and Lauren, 2018; World Wildlife Fund, 2019). To address these issues, the FAO proposed indicators and practices to perform sustainability assessments of food and agriculture systems (SAFA), including economic, social, governance and

environmental aspects. There are some important environmental indicators involved in this thesis such as GHG reduction and mitigation practices, air and water pollution, and nutrient balance while applying animal, soil, and water bodies health practices (FAO, 2013). To reduce those impacts, strategies modifying the diet of dairy cows, improving feed efficiency, to reduce nitrogen accumulation in manure, and gas emissions can be applied. Feed efficiency in dairy cows is affected importantly by forage quality and digestibility, mainly because they are a large part of the rations (Oenema and Oenema, 2022). Animal health, ration composition, lactation stage and maintenance requirements of the herds, are also factors to take into account, as they regulate how much feed energy intake will be transformed into milk production (Brito and Silva, 2020). Improving feed efficiency has been found a way to reduce farm costs and environmental impacts. Therefore, a better knowledge of nutritional cattle physiology and nutrient requirements have conducted to better diet formulation, supplementation, processing and storage techniques (De Ondarza and Tricarico, 2017).

Traditionally, dairy cows were fed a concentrate feed in the milking parlor. However, with the introduction of total mixed rations (TMR), a mix of forages and grains providing all of the cow's nutrient requirements, the practice to feed animals in the milking parlor has been progressively abandoned. The introduction of TMR was a revolution in the feeding and management of dairy cows. Feeding cows with TMR increased milk yield because cows were fed a homogenous diet more adapted to their nutrient requirements, and it also improved diet management with the use of wagon mixers. However, dairy cows need different amounts of protein depending on their physiological status and milking performance while TMR's diet is designed for a reference cow. Therefore, some cows in the group receive more and others fewer nutrients than they really need (Bach, 2014). Moreover, as lactation advances, protein requirements decrease so, in late lactation, the urinary and fecal nitrogen excreta may increase if the diet is not adjusted (Law *et al*., 2009) contributing to a major emission of NH_3, which is volatilized mostly during manure storage (Kupper *et al*., 2020).

To minimize these deviations, some alternatives were introduced in the 1980s, such as automatic concentrate feeders in automatic milking system (AMS). The AMS depends to a great extent on supplementing cows with a fixed amount of concentrate feed to motivate the cows to visit the system and minimize the number of cows that need to be encouraged to go to the AMS (Bach *et al*., 2007; Halachmi *et al*., 2005). Recently, particularly in South Africa and New Zealand, the rotatory milking parlors have adopted a new technology that allows mixing two different feeds and offering a "personalized" supplement for each cow based on their milk production and change of body weight (BW) if the facility is equipped with a BW scale (Bach, 2014). Nowadays, technology has advanced, and some milking equipment can measure milk components such as fat and protein, improving the accuracy of nutrient requirements estimation of each individual cow

(Bach, 2014). Using such nutritional strategies that allow to feed animals according to their production level and nutrient requirements is known as precision feeding.

Another parallel approach to reduce the concentration of nitrogen in cow manure is treating it from a microbiological point of view. A major part of crude protein is degraded to NH_3 in the rumen, which accounts for the main nitrogen source for microorganisms. An excessive decomposition of protein by proteolysis and deamination produces high amounts of NH_3, which will be converted to urea and excreted in urine and manure (Attwood *et al*., 1998; Eschenlauer *et al*., 2002; Hartinger *et al*., 2018).

Protein degradation (soluble protein) by bacteria in the rumen follows the path presented in Figure 3.

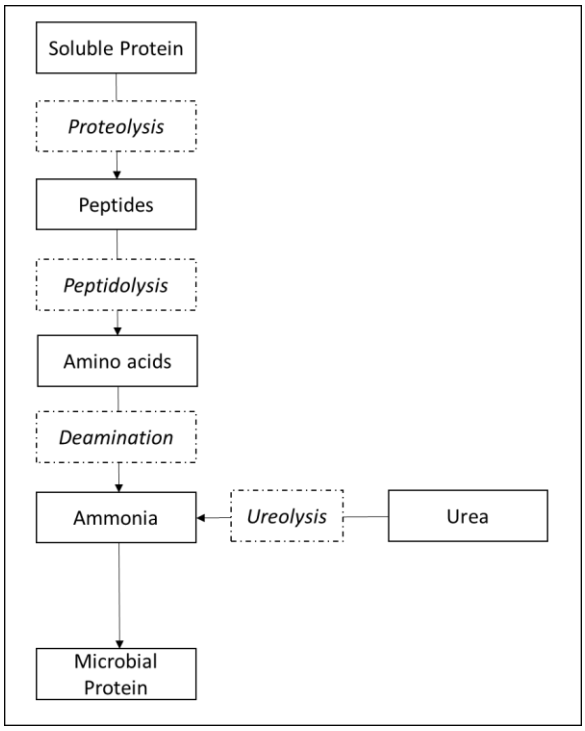


Figure 3. Soluble protein degradation in rumen. Adaptation from Hartinger *et al*. (2018).

Generally, an increase of NH_3 production is observed when high soluble protein levels are fed to cattle, but Attwood *et al*. (1998) also identified hyper ammonia-producing bacteria (HAP), deaminating bacteria that produce a high quantity of NH_3 (*Clostridium sticklandii*, *Clostridium aminophilum*, and *Peptostreptococcus anaerobius*), favoring nitrogen/ammonia accumulation/inefficiency in the rumen.

Several studies were done to decrease HAP population by introducing some plants or extracts to the diet, such as tamarind and tannins (Tan *et al*., 2021), or hops and spent yeast (Flynthe *et al*., 2017) that had antimicrobial effects against them. In the recent years, bacteriophages, virus/phages that infect and replicate within bacteria and archaea, have been used as indicators of

fecal contamination and as antimicrobials, due to their ability to infect specific bacteria species or even specific strains (Yosef *et al*., 2014). They are appearing to be a new alternative therapy to control bacteria in plants, animals, food, and humans, and they are common in all natural environments (Gul and Alsayeqh, 2022). In this sense, another strategy to reduce HAP population in the rumen could be based on the use of specific rumen bacteriophages against HAP. They are found in every environment, and they are considered the most abundant biological agent on Earth (Kasman and Porter, 2022). Gilbert *et al*. (2017) performed a genome study of bacteriophages present in the rumen, and they identified bacteriophages against *Bacteroides*, *Ruminococcus*, and *Streptococcus* isolated from ruminal fluid, and Moodley *et al*. (2019), managed to isolate a specific bacteriophage of *Staphylococcus pseudintermedius*.

Manure valorization technologies

Circular agriculture focuses on using minimal amounts of external inputs, closing nutrient loops, regenerating soils, and minimizing the impact on the environment, thus contributing to stimulate the transition towards sustainable and resilient energy and farming systems (Helgason *et al*., 2021). Using manure as a source of macro and micronutrients in agriculture is a practice that allows crop and livestock production without the depletion of non-renewable sources and without harming the environment by, for instance, reducing the dependence on mineral and synthetic fertilizers (Prado *et al*., 2022). In Europe, it is estimated that a circular approach to food systems could reduce the use of chemical fertilizers by 80% (Helgason *et al*., 2021). However, if not managed properly, the excess application of manure to soil can lead to detrimental effects on the environment due to ammonia emissions, pollution of surface water by run-off and groundwater by NO_3^- leaching. Thus, the application of manure and by-products to soil in the EU is strictly regulated (maximum 170 kg N ha^{-1} $year^{-1}$ in Nitrate Vulnerable Zones), considering the environmental risks associated with nitrate diffuse pollution (European Commission, 1991). However, more than 90% of manure produced in the EU27 is currently returned to agricultural fields, representing about 53% of the P and 33% of the N applied annually to agricultural soil (NRR, 2016).

Soil application is a controversial management form, as it generates dangerous liquids with bacteria and chemical contaminants as well as being a potential source of emission of volatile compounds that contribute to climate change and atmospheric pollution (Pawelczyk, 2005). Manure processing is defined as treating manure in such a way that the N present is not brought back onto the soil after treatment so that it is either exported or converted to nitrogen gas or a mineral fertilizer (Lebuf *et al*., 2012). Livestock manure has been treated by separation, use of additives (acid, lime, temperature/pressure), air cleaning (scrubbing, biofiltration and

bioscrubbing), and mainly anaerobic digestion (AD), to produce energy and digestate to be used in the field as a fertilizer (Foged *et al*., 2011). In the AD, although the digestates composition varies strongly according to the feedstock composition, digester type, and process parameters, a fraction of the complex organic matter (OM) always remains (Vaneeckhaute *et al*., 2017), while the easily degradable OM is converted into CH_4 and CO_2. Thereby, an amount of effective organic carbon (OC) present in digestates can be applied to soils contributing to the humus built-up. Moreover, organically bound N and P are released as ammonium (NH_4^+) and phosphate, which are directly available for crop uptake: the higher the share or soluble easy available molecules, the higher the efficiency of the digestate as fertilizer. Also, the total contents of K, Ca, Mg, and heavy metals are not altered during AD, while weed seeds and pathogens can be killed off during the process, depending on the temperature and residence time in the digester and on the type of organism (Vaneeckhaute *et al*., 2012; Vaneeckhaute *et al*., 2013; Vaneeckhaute *et al*., 2014; Moeller and Mueller, 2012).

Digestate nutrient richness can produce gas emission and leaching, so it must be treated before land application to reduce losses (Ma *et al*., 2018). The selection of the nutrient recovery technology (Figure 4) depends on the input waste stream characteristics and has a strong influence on the composition and properties of the resulting fertilizer end-product and by-products (Vaneeckhaute *et al*. 2017; Angouria-Tsorochidou *et al*., 2022). To achieve this recovery, digestates are usually submitted to a liquid-solid separation process. The main common technologies are divided by gravity (statics, vibratory and rotatory sieves), pressure (screw press and filter belts), and centrifugation (decanting centrifuges) (Bonmatí *et al*., 2020). Although mechanical separation creates an end-product (i.e., the solid fraction) with a higher nutrient concentration than the raw digestate, it is not considered a nutrient recovery technique because it is merely the first step for further processing (Lebuf *et al*., 2012).

To be considered as organic fertilizer and labeled to be marketable within the European Union, the final product must fulfill the European regulation of fertilizers (Regulation EU 2019/1009). In general, NPK solid organic fertilizers must have a concentration of Total Nitrogen (TN) > 1% of total solids (TS), P_2O_5 > 1% TS, K_2O > 1% TS, NPK > 4% TS, and Total Organic Carbon (TOC) > 15% TS. For a solid organic fertilizer declaring only a primary nutrient, the required concentrations are TN > 2.5% TS, or P_2O_5 > 2% TS, or K_2O > 2% TS. The difference with liquid organic fertilizer is that, for the liquid, the TOC should be at least 5% TS.

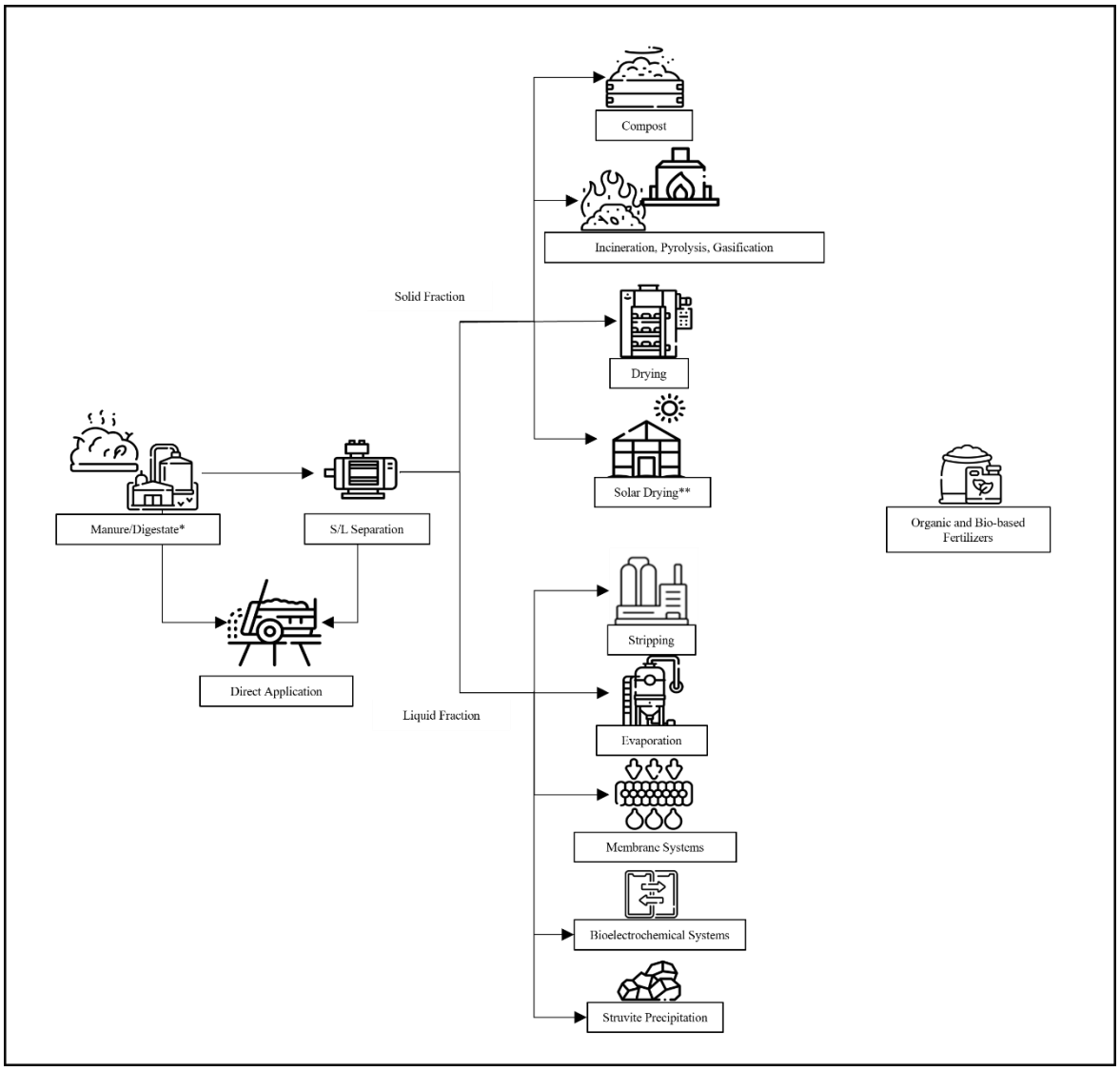


Figure 4. Main manure treatment technologies to produce organic and BBF. *The same technologies can be applied to both manure or digested manure, being the digestate more stable and releasing less emissions. **Discussed in this thesis.

The liquid fraction of digestate can receive different post-treatments depending on the final objective, remove nutrients or concentrate them to produce fertilizers. Membrane separation, reverse osmosis, evaporation, or a combination of various technologies are suitable to concentrate specific compounds in small volumes, such as N and P (Angouria-Tsorochicou *et al.*, 2022; Duttas *et al*., 2021). Filtration can be performed to feed microalgae for protein production (Seelam *et al*., 2022). The liquid fraction can be treated as well by stripping to concentrate NH_3 or crystallized as struvite to obtain a phosphorus-based fertilizer (Ma *et al*., 2018). For the recovery of phosphorus, as calcium phosphate, the use of upflow anaerobic sludge blank (UASB) reactors (Tervahauta *et al.*, 2014) and anaerobic ammonium oxidation (Anammox) (Johansson *et al*., 2017) had been studied.

Then, the solid part is treated by drying, incineration, or composting processes to facilitate their transport, stabilization of its OM and hygienisation, although it can be applied directly as a fertilizer. Many facilities have found that drying the raw digestate or the solid fraction is an economically viable way because the end-product has reduced volume, making it suitable for exportation (Lebuf *et al*., 2012). The problem with thermal drying and incineration is their high energy consumption, as drying costs for sludges are reported as 2635 kW/ton (Li *et al.*, 2016). In most cases, close AD plants often can cope with a considerable amount of excess heat from the combustion-heat power (CHP) engine that can be used for drying purposes, for heating the digester itself, and /or for covering the thermal energy demand of nearby stables or houses. Moreover, a nutrient loss is sometimes reported; for example, composting, even when correctly performed, releases emissions into the atmosphere of 113-127 g NH_3/kg TN, 37-46 g N_2O/kg TN, and 1.0-1.9 g CH_4/kg OM (Fukumoto *et al*., 2003).

Opposite to conventional thermal dryers, solar dryers have been used as a traditional method for food preservation (Jairaj *et al*., 2009). In countries with high solar radiation, it is an energetically sustainable method since it uses energy from the sun (Ndukwu *et al*., 2018). In 2005, the "Institute of Heat Engineering, Warsaw University of Technology" developed the concept of solar dryer for wastewater, in which energy used was lower than in other drying facilities (Krawczyk *et al*., 2011) and nowadays solar drying for sewage sludge is a reality, with companies developing solar drying treatment systems at full-scale (Helantis, 2022). Prenafeta-Boldú *et al.* (2020, 2021) dewatered sewage sludge from a wastewater treatment plant of winery residues and pig slurry, respectively, with a pilot-scale greenhouse solar dryer. Recently, Battista and Bolzonella (2019) studied the solar drying of digested slurry for the recovery of ammonium sulfate, employing a solar dryer with a greenhouse configuration at the laboratory scale, promoting the use of solar energy in the treatment of waste. However, emission control during the drying process, scaling-up (and all the modification of the system that represents) and valorizing the dried product need to be considered.

References

Angouria-Tsorochidou, E., Seghetta, M., Trémier, A., Thomsen, M. 2022. Life cycle assessment of digestate post-treatment and utilization. Science of the Total Environment, 815. https://doi.org/10.1016/j.scitotenv.2021.152764.

Arcas-Pilz, V., Parada, F., Rufí-Salis, M., Stringari, G., González, R., Villalba, G., Gabarrell, X. 2022. Extended use and optimization of struvite in hydroponic cultivation systems. Resources, Conservation and Recycling, 179. https://doi.org/10.1016/j.resconrec.2021.106130.

Arrobas, M., Decker, J.V., Feix, B.L., Godoy, W.I., Casali, C.A., Correia, C.M., Ângelo Rodrigues, M. 2022. Biochar and zeolites did not improve phosphorus uptake or crop productivity in a field trial performed in an irrigated intensive farming system. Soil Use and Management, 38(1), 564–575. https://doi.org/10.1111/sum.12704.

Attwood, G.T., Klieve, A.V., Ouwerkerk, D., Patel, B.K.C. 1998. Ammonia-Hyperproducing bacteria from New Zealand ruminants, 1998. Applied and Environmental Microbiology, pp. 1796-1804.

Bach, A. 2014. Precision Feeding to increase efficiency for milk production. WCDS Advances in Dairy Technology (2014) Volume 26: 177 – 189.

Bach, A., Iglesias, C., Calsamiglia, S., Devant, M. 2007 Effect of amount of concentrate offered in automatic milking systems on milking frequency, feeding behavior, and milk production of dairy cattle consuming high amounts of corn silage J. Dairy Sci. 90:5049–5055.

Battista, F., Bolzonella, D. 2019. Exploitation of Solar Energy for Ammonium Sulfate Recovery from Anaerobic Digestate of Different Origin. Waste and Biomass Valorization, 10(12), 3701–3709. https://doi.org/10.1007/s12649-019-00597-x.

Bergstrand, K.J. 2022. Organic fertilizers in greenhouse production systems – a review. In Scientia Horticulturae (Vol. 295). Elsevier B.V. https://doi.org/10.1016/j.scienta.2021.110855.

Bonmatí-Blasi, A., Cerrillo-Moreno, M., Riau-Arenas, V. 2020. Systems Based on Physical-Chemical Processes: Nutrient Recovery for Cycle Closure. In I. Management Association (Ed.), Waste Management: Concepts, Methodologies, Tools, and Applications (pp. 526-558). IGI Global. https://doi.org/10.4018/978-1-7998-1210-4.ch026.

Brito, A. F., Silva, L. H. P. 2020. Symposium review: Comparisons of feed and milk nitrogen efficiency and carbon emissions in organic versus conventional dairy production systems. J. Dairy Sci. 103, 5726–5739. doi: 10.3168/jds.2019-17232.

Burning, H. 2022. Dairy Farming: Environmental Impact, Ethics, and Industry Outlook. How much does dairy farming contribute to global warming? Treehugger Sustainable for all. Available online: https://www.treehugger.com/dairy-farming-environmental-impact-5323217#citation-19. Accessed November 2022.

De Ondarza, M. B., Tricarico, J. 2017. REVIEW: Advantages and limitations of dairy efficiency measures and the effects of nutrition and feeding management interventions. Prof. Anim. Sci. 33, 393–400. doi: 10.15232/pas.2017-01624.

Directive of the Council of 12 December 1991 concerning the protection of waters against pollution caused by nitrates from agricultural sources (91/676/EC).

Dutta, S., He, M., Xiong, X., Tsang, D. C. W. 2021. Sustainable management and recycling of food waste anaerobic digestate: A review. In Bioresource Technology (Vol. 341). Elsevier Ltd. https://doi.org/10.1016/j.biortech.2021.125915.

Eschenlauer, S.C.P., McKain, N., Walker, N.D., McEwan, N.R., Newbold, C.J., Wallace, R.J. 2002. Ammonia production by ruminal microorganisms and enumeration, isolation, and characterization of bacteria capable of growth on peptides and amino acids from the sheep rumen. Applied and Environmental Microbiology, pp. 4925-4931.

EC. 2019. Communication from the commission to the European parliament, the European council, the council, the European economic and social committee and the committee of the regions. The European Green Deal. COM/2019/640 final. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A52019DC0640.

EC. 2020. "A Farm to Fork Strategy for a Fair, Healthy and Environmentally-Friendly Food System." COM(2020) 381 Final. Brussels: European Commission. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52020DC0381.

EC 2021. EU agricultural outlook for markets, income and environment, 2021-2031. European Commission, DG Agriculture and Rural Development, Brussels.

Eurostat. 2023. Agri-environmental indicator – livestock patterns. Available online: https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Agri-environmental_indicator_-_livestock_patterns#Livestock_density_at_EU_level_in_2020 (Accessed on April 2023).

Fang, P., Abler, D., Lin, G., Sher, A., Quan, Q. 2021. Substituting organic fertilizer for chemical fertilizer: Evidence from apple growers in china. Land, 10(8). https://doi.org/10.3390/land10080858.

FAO. 2009. Livestock in the balance THE STATE OF FOOD AND AGRICULTURE. http://www.fao.org/catalog/inter-e.htm.

FAO. 2013. SAFA Sustainability Assessment of Food and Agriculture Systems. Guidelines, version 3.0. Food and Agriculture Organization, Rome, 253 p., http://www.fao.org/nr/sustainability/sustainabilityassessments-safa/en/.

FAOSTAT. 2022. https://ourworldindata.org/fertilizers. Data from: http://www.fao.org/faostat/en/#data. Consulted October 2022.

FASUSDA. 2022. Foreign Agricultural Service of the United States Department of Agriculture. Impacts and Repercussions of Price Increases on the Global Fertilizer Market Prepared By: Global Market Analysis, Modeling Team.

Flythe, M.D., Harlow, B.E., Aiken, G.E., Gellin, G.L., Kagan, I.A., Pappas, J. 2017. "Inhibition of Growth and Ammonia Production of Ruminal HAP by Chinook or Galena Hops after Long-Term Storage" Fermentation 3, no. 4: 68. https://doi.org/10.3390/fermentation3040068.

Foged, H.L., Flotats, X., Bonmati, A., Palatsi, J., Magri, A.,Martin Schelde, K. 2011. Inventory of manure processing activities in Europe. Technical Report No. I concerning "Manure Processing Activities in Europe" to the European Commission, Directorate-General Environment. 138 pp.

Fukumoto, Y., Osada, T., Hanajima, D., & Haga, K. 2003. Patterns and quantities of NH3, N2O and CH4 emissions during swine manure composting without forced aeration–effect of compost pile scale. Bioresource Technology, 89(2), 109-114.

Gerber, P.J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A., Tempio, G. 2013. Food and Agriculture Organization of the United Nations.Tackling climate change through livestock: a global assessment of emissions and mitigation opportunities. ISBN 978-92-5-107920-1.

Gilbert, R.A., Kelly, W.J., Altermann, E., Leahy, S.C., Minchin, C., Ouwerkerk, D., Klieve, A.V. 2017. Toward Understanding Phage:Host Interactions in the Rumen; Complete Genome Sequences of Lytic Phages Infecting Rumen Bacteria. Front. Microbiol. 8:2340.

Gul, S.T., Alsayeqh, A.F. Probiotics as an alternative approach to antibiotics for safe poultry meat production. Pak Vet J. (2022) 42. doi: 10.29261/pakvetj/2022.061.

Halachmi, I., S. Ofir, and J. Miron. 2005. Comparing two concentrate allowances in an automatic milking system. Animal Science Journal. 80:339–343.

Helantis natural sludge drying. https://www.suezwaterhandbook.com/degremont-R-technologies/sludge-treatment/drying/natural-sludge-drying-in-green-house-Heliantis. Accessed October 2022.

Helgason, K., Iversen, K., Julca, A. 2021. Development Research Branch, Economic Analysis and Policy Division, UN DESA. This Policy Brief is based on World Social Report 2021: Reconsidering Rural Development. https://www.un.org/development/desa/dpad/publication/un-desa-policy-brief-105-circular-agriculture-for-sustainable-rural-development/0.

Jairaj, K.S., Singh, S.P., Srikant, K. 2009. A review of solar dryers developed for grape drying. Solar Energy, 83, 1698-1712.

Johansson, S., Ruscalleda, M., Colprim, J. 2017. Phosphorus recovery through biologically induced precipitation by partial nitritation-anammox granular biomass. Chemical Engineering Journal, 327, pp. 881-888.

Kasman, L.M., Porter, L.D. 2022. Bacteriophages. Sep 26. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan–. PMID: 29630237.

Krawczyk, P., Badyda, K. 2011. Modeling of thermal and flow processes in a solar waste-water sludge dryer with supplementary heat supply from external sources. Journal of Power Technologies 91 (1) 37–4.

Kyriakou, V., Garagounis, I., Vourros, A., Vasileiou, E., Stoukides, M. 2020. An Electrochemical Haber-Bosch Process. Joule, 4(1), 142–158. https://doi.org/10.1016/j.joule.2019.10.006.

Koul, B., Yakoob, M., & Shah, M. P. 2022. Agricultural waste management strategies for environmental sustainability. Environmental Research, 206. https://doi.org/10.1016/j.envres.2021.112285.

Kupper, T., C. Häni, A. Neftel, C. Kincaid, M. Bühler, B. Amon, and A. VanderZaag. 2020. NH3 and greenhouse gas emissions from slurry storage – A revie., Agric. Ecosyst. Environ. 300:106963. https://doi.org/10.1016/j.agee.2020.106963.

Law, R. A., F. J. Young, D. C. Patterrson, D. J. Kilpatrick, A. R. G. Wylie, and C. S. Mayne. 2009. Effect of dietary protein content on animal production and blood metabolites of dairy cows during lactation. J. Dairy Sci. 92:1001-1012. https://doi.org/10.3168/jds.2008-1155.

Lebuf, V., Accoe, F., Vaneeckhaute, C., Meers, E., Michels, E., Ghekiere, G. 2012. Nutrient recovery from digestates: techniques and end-products. In Proceedings of IV International Symposium on Energy from Biomass and Waste, Venice.

Li, B., Wang, F., Chi, Y., Yan, J.H. 2014. Study on optimal energy efficiency of a sludge drying-incineration combined system. J Mater Cycles Waste Manag 16, 684–692. https://doi.org/10.1007/s10163-014-0293-3.

Li, C. Sheng, C., Rong, R.. 2022. Preparation of solid organic fertilizer by co-hydrothermal carbonization of peanut residue and corn cob: A study on nutrient conversion. Science of the Total Environment, 838. https://doi.org/10.1016/j.scitotenv.2022.155867.

Loss, A., Couto, R. da R., Brunetto, G., Veiga, M. da, Toselli, M., Baldi, E. 2019. Animal manure as fertilizer: changes in soil attributes, productivity and food composition. International Journal of Research -GRANTHAALAYAH, 7(9), 307–331. https://doi.org/10.29121/granthaalayah.v7.i9.2019.615.

Ma, H., Guo, Y., Qin, Y., Li, Y. 2018. Nutrient recovery technologies integrated with energy recovery by waste biomass AD. Bioresource Technology, 269: 520-531.

Menardo, S., Lanza, G., Berg, W. 2021. The effect of diet and farm management on n2o emissions from dairy farms estimated from farm data. Agriculture (Switzerland), 11(7). https://doi.org/10.3390/agriculture11070654.

Merrill, D., Lauren, L. 2018. "Here's How America Uses Its Land." Bloomberg. Available online: https://www.bloomberg.com/graphics/2018-us-land-use/. Accessed November 2022.

Moeller, K., Mueller, T. 2012. Effects of AD on digestate nutrient availability and crop growth: a review. Eng.Life Sci. 12(3), 242–257.

Moodely, A., Kot, W., Nälgard, S., Jakociune, D., Neve, H., Hansen, L.H., Guardabassi, L., Vogensen, F.K. 2019. Isolation and characterization of bacteriophages active against methicillin-resistant Staphylococcus pseudintermedius. Research in Veterinary Science, 122:81-85.

Mózner, Z., Tabi, A., Csutora, M. 2012. Modifying the yield factor based on more efficient use of fertilizer – The environmental impacts of intensive and extensive agricultural practices. Ecological Indicators, 16, 58–66. https://doi.org/10.1016/j.ecolind.2011.06.034.

Ndukwu, M.C., Bennamoun, L., Abam, F. I. 2018. Experience of solar drying in Africa: Presentation of designs, operations and models. Food Engineering Reviews, December 2018, Volume 10, Issue 4, pp 211–244.

Nutrient Recovery and Reuse (NRR) in European agriculture. A review of the issues, opportunities, and actions. 2016. RISE Foundation.

OECD, F.A.O.U.N., 2018. OECD-FAO Agricultural Outlook 2018-2027. https://doi.org/10.1787/agr_outlook-2018-en.

Oenema, J., Oenema, Oene. 2022. Unraveling feed and nutrient use efficiencies in grassland-based dairy farms. Front. Sustain. Food Syst., 29 September 2022. Sec. Waste Management in Agroecosystems. Volume 6 - 2022 | https://doi.org/10.3389/fsufs.2022.846561.

Pavwelczyk, A. 2005. EU Policy and Legislation on recycling of organic wastes to agriculture. International Society for Animal Hygiene. 1.

Peyraud, J.L., MacLeod, M., Europäische Kommission Generaldirektion Landwirtschaft und Ländliche Entwicklung. 2020. Study on future of EU livestock how to contribute to a sustainable agricultural sector? Final report.

Prado, J., Ribeiro, H., Alvarenga, P., Fangueiro, D. 2022. A step towards the production of manure-based fertilizers: Disclosing the effects of animal species and slurry treatment on their nutrients content and availability. J Cleaner Prod. 337. https://doi.org/10.1016/j.jclepro.2022.130369.

Prenafeta-Boldú, F. X., Burgos, L., Noguerol, J., Fernández, B., Mercader, M., Soler, J. 2020. Solar drying in the vineyard: A sustainable technology for the recovery of nutrients from winery organic waste. Water Science and Technology, 82(1), 27–38. https://doi.org/10.2166/wst.2020.320.

Prenafeta-Boldú, F. X., Fernández, B., Viñas, M., Noguerol, J., Soler, J., & Illa, J. 2021. Combined acidification and solar drying of pig slurries for nutrient recovery and controlled atmospheric emissions. Agronomy, 11(2). https://doi.org/10.3390/agronomy11020222.

Prosser, J. I., Hink, L., Gubry-Rangin, C., Nicol, G. W. 2020. Nitrous oxide production by ammonia oxidizers: Physiological diversity, niche differentiation and potential mitigation strategies. In Global Change Biology (Vol. 26, Issue 1, pp. 103–118). Blackwell Publishing Ltd. https://doi.org/10.1111/gcb.14877.

Rasouli, F., Nasiri, Y., Asadi, M., Hassanpouraghdam, M. B., Golestaneh, S., Pirsarandib, Y. 2022. Fertilizer type and humic acid improve the growth responses, nutrient uptake, and essential oil content on Coriandrum sativum L. Scientific Reports, 12(1). https://doi.org/10.1038/s41598-022-11555-4.

Ritchie, H., Roser, M., Rosado, P. 2022. “Fertilizers”. Published online at OurWorldInData.org. Retrieved from: ‘https://ourworldindata.org/fertilizers’. Accessed: April 2023.

Seelam, J.S., Fernandes de Souza, M., Chaerle, P., Willems, B., Michels, E., Vyverman, W., Meers, E. 2022. Maximizing nutrient recycling from digestate for production of protein-rich microalgae for animal feed application. Chemosphere, 290, Article 133180, 10.1016/j.chemosphere.2021.133180.

Sigurnjak, I., Michels, E., Crappé, S., Buysens, S., Biswas, J. K., Tack, F.M.G., de Neve, S., Meers, E. 2017. Does acidification increase the nitrogen fertilizer replacement value of bio-based fertilizers? Journal of Plant Nutrition and Soil Science, 180(6), 800–810. https://doi.org/10.1002/jpln.201700220.

Swoboda, P., Döring, T.F., Hamer, M. 2022. Remineralizing soils? The agricultural usage of silicate rock powders: A review. In Science of the Total Environment (Vol. 807). Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2021.150976.

Tan, P., Liu, H., Zhao, J., Gu, X., Wei, X., Zhang, X., Ma, N., Johnston, L.J., Bai, Y., Zhang, W., Nie, C., Ma, X. 2021. Amino acids metabolism by rumen microorganisms: Nutrition and ecology strategies to reduce nitrogen emissions from the inside to the outside, Science of The Total Environment, Volume 800, 149596, ISSN 0048-9697, https://doi.org/10.1016/j.scitotenv.2021.149596.

Ti, C., Xia, L., Chang, S. X., Yan, X. 2019. Potential for mitigating global agricultural ammonia emission: A meta-analysis. Environmental Pollution, 245, 141–148. https://doi.org/10.1016/j.envpol.2018.10.124.

Tervahauta, T., van der Weijden, R.D., Flemming, R.L., Hernández Leal, L., Zeeman, G., Buisman, C.J.N. 2014. Calcium phosphate granulation in anaerobic treatment of black water: a new approach to phosphorus recovery. Water Resource, 48, pp. 632-642.

Tripathi, N., Hills, C.D., Singh, R.S., Atkinson, C.J. 2019. Biomass waste 18eteorologi in low-carbon products, harnessing a major potential resource. Clim. Atmos. Sci. 2 (1), 1–10.

United Nations, Department of Economic and Social Affairs, Population Division 2022. World Population Prospects 2022: Summary of Results. UN DESA/POP/2022/TR/NO. 3.

Uwizeye, A., de Boer, I. J. M., Opio, C. I., Schulte, R. P. O., Falcucci, A., Tempio, G., Teillard, F., Casu, F., Rulli, M., Galloway, J. N., Leip, A., Erisman, J. W., Robinson, T. P., Steinfeld, H., Gerber, P. J. 2020. Nitrogen emissions along global livestock supply chains. Nature Food, 1(7), 437–446. https://doi.org/10.1038/s43016-020-0113-y.

VanderZaag, A.C., Gordon, R.J., Jamieson, R.C., Burton, D.L., Stratton, G.W. 2009. Gas emissions from straw covered liquid dairy manure during summer storage and autumn agitation. Transactions of the ASABE, 52 (2), pp. 599-608.

Vaneeckhaute, C., Meers, E., Michels, E., Christiaens, P., Tack, F.M.G. 2012. Fate of macronutrients in water treatment of digestate using vibrating reversed osmosis. Water Air Soil Poll. 223(4), 1593–1603.

Vaneeckhaute, C., Meers, E., Michels, E., Ghekiere, G., Accoe, F., Tack, F.M.G. 2013. Closing the nutrient cycle by using bio-digestion waste derivatives as synthetic fertilizer substitutes: a field experiment. Biomass Bioenerg 55, 175–189.

Vaneeckhaute, C., Ghekiere, G., Michels, E., Vanrolleghem, P.A., Tack, F.M.G., Meers, E. 2014. Assessing nutrient use efficiency and environmental pressure of macro-nutrients in bio-based mineral fertilizers: a review of recent advances and best practices at field scale. Adv. Agron. 128, 137–180.

Vaneeckhaute, C., Lebuf, V., Michels, E., Belia, E., Vanrolleghem, P.A., Tack, F.M.G., Meers, E. 2017. Nutrient Recovery from Digestate: Systematic Technology Review and Product Classification. Waste Biomass Valorization, 2017. 8, 21–40.

Walling, E., Vaneeckhaute, C. 2020. Greenhouse gas emissions from inorganic and organic fertilizer production and use: A review of emission factors and their variability. In Journal of Environmental Management (Vol. 276). Academic Press. https://doi.org/10.1016/j.jenvman.2020.111211.

World Wildlife Fund. 2019. Milk's Impact on the Environment. Available online: https://www.worldwildlife.org/magazine/issues/winter-2019/articles/milk-s-impact-on-the-environment. Accessed November 2022.

Yosef, I., Kiro, R., Molshanski-Mor, S., Edgar, R., Qimron, U. 2014. Different approaches for using bacteriophages against antibiotic-resistant bacteria. Bacteriophage. 4:e28491. doi: 10.4161/bact.28491.

Yüzbaşıoğlu, A. E., Avşar, C., Gezerman, A. O. 2022. The current situation in the use of ammonia as a sustainable energy source and its industrial potential. In Current Research in Green and Sustainable Chemistry (Vol. 5). Elsevier B.V. https://doi.org/10.1016/j.crgsc.2022.100307.

Zhang, N., Bai, Z., Ledgard, S., Luo, J., Ma, L. 2021. Ammonia mitigation effects from the cow housing and manure storage chain on the nitrogen and carbon footprints of a typical dairy farm system on the North China Plain. Journal of Cleaner Production, 280. https://doi.org/10.1016/j.jclepro.2020.124465.

Zhang, N., Bai, Z., Winiwarter, W., Ledgard, S., Luo, J., Liu, J., Guo, Y., Ma, L. 2019. Reducing ammonia emissions from dairy cattle production via cost-effective manure management techniques in China. Environmental Science and Technology. https://doi.org/10.1021/acs.est.9b04284.

Chapter 2 – Objective and outline of the thesis

Objective

The general objective of this thesis is to contribute to the improvement of the environmental sustainability of intensive livestock systems, by applying innovative strategies to close nutrient cycles and reduce pollutant emissions at farm and manure management level. This wider objective can be divided into three specific objectives (SO):

SO1. To improve nutrient use and recovery at animal production level.

- Adjusting energy and protein requirements in dairy farms to avoid unnecessary losses.

SO2. To reduce GHG and NH_3 emissions during manure storage and treatment.

- Adjusting energy and protein requirements in dairy farms to reduce gas emissions in manure.
- Modifying dairy cows' ruminal microbiome responsible for NH_3 emissions.
- Acidifying digestate and derivates during a fertilizing production process to reduce gas emissions.

SO3. To increase the fertilizer value of livestock manure and anaerobic digestate from agro-industrial biogas plants.

- Acidifying digestate and derivates during a fertilizing production process to retain nitrogen.
- Testing the resulting fertilizers to determine their phytotoxicity and plant growth.

Thesis outline

This thesis is structured in 9 chapters (Figure 5). A general and a wide vision of the state of the art, the main challenges that this thesis tries to overcome and the objectives of this work are presented in **Chapter 1 – Introduction**.

Chapter 3 defines the legal framework in which the thesis had been developed, including the main environmental regulations that affect the experiments and how they are approached throughout the thesis.

Chapter 4 describes the different locations of the experimental set-ups, their main characteristics and a brief description of the principal innovations developed.

The first experimental study of this work is presented in **Chapter 5 – "Effectiveness of precision feeding in reducing N excretion in dairy cattle"**. It shows the effect of precision feeding application on dairy cows to optimize nitrogen efficiency through the diet, reducing nitrogen

excretion in urine. In addition, GHG and NH_3 emissions were monitored during manure storage to see precision feeding effect on manure storage emissions.

Chapter 6 – "Identification of bacteriophages linked to the activity of ruminal hyper-ammonia producing bacteria (HAP)" introduces the isolation and identification of hyper-ammonia producing bacteria from the ruminal fluid of dairy cows, and the techniques used to inoculate bacteriophages that can interact with them, reducing their population.

Chapter 7 - "Acidification and solar drying of manure-based digestate to produce improved fertilizing products" describes the application of an innovative manure and digestate valorization chain to produce different bio-based fertilizers composed of a solar drying greenhouse, a stripping/scrubbing unit and the use of acidifying agents to reduce emissions. Furthermore, this chapter compares the produced fertilizers with the current legislation of organics fertilizers and proves their viability with an experimental pot trial with lettuce seedlings.

The last experimental chapter, **Chapter 8 – "Germination and growth response of lettuce (Lactuca sativa) under greenhouse conditions fertilized by novel biobased fertilizing products derived from pig manure"**, validates the fertilizing potential of solar-dried digestate from pig and dairy manure in lettuce seed germination and plant pot assays.

Finally, in **Chapter 9**, a wrap-up discussion is presented about all the explored topics during the thesis, followed by general conclusions and future perspectives.

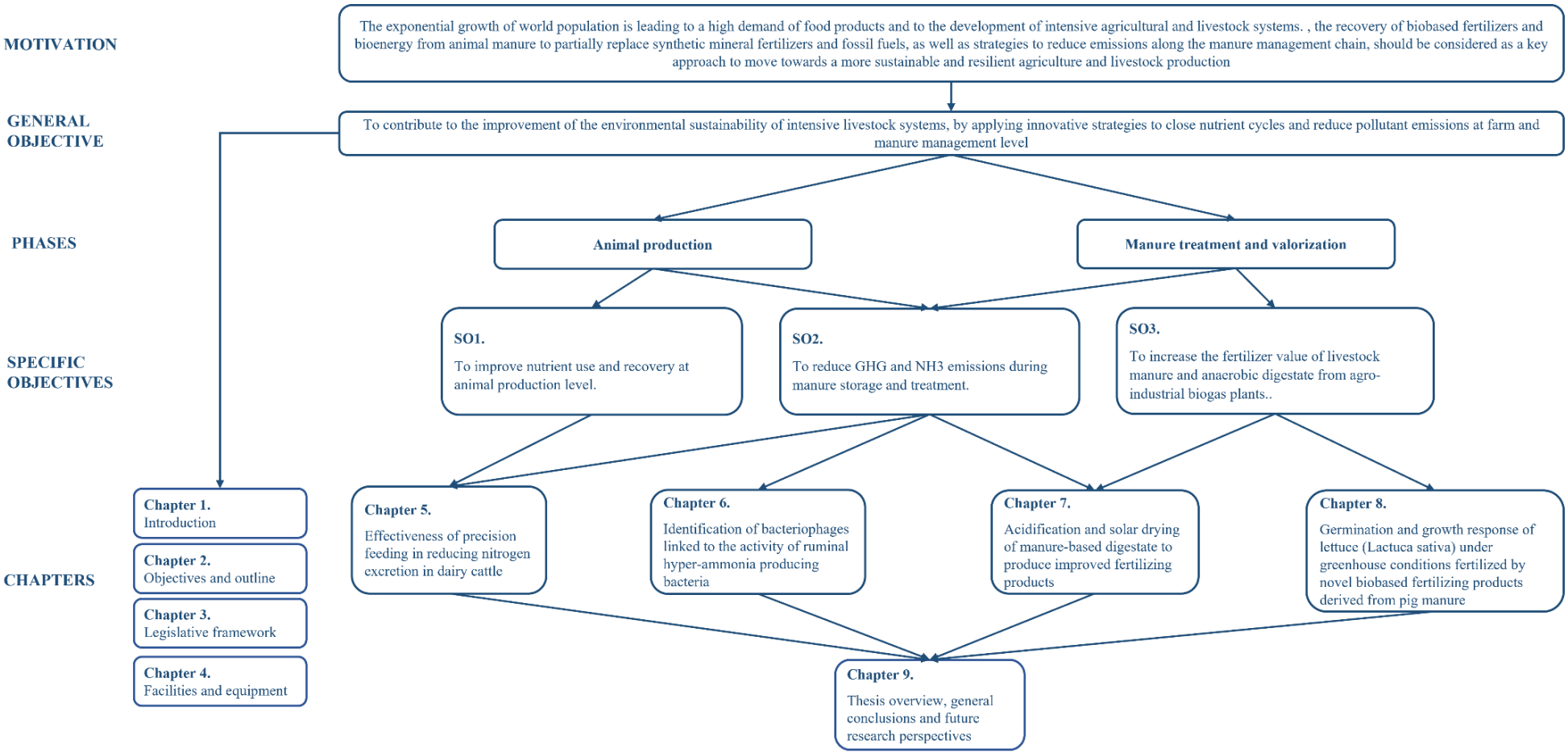


Figure 5. Thesis outline scheme.

Chapter 3 – Legislative Framework

Agriculture, Waste, Emissions to the environment and Circular economy EU legislations were chosen through all the environmental policies as the most related to contextualize the regulation framework of this thesis. The main related regulations within these groups are presented and connected to how they interact with each part of the thesis to increase circularity and sustainable nutrient use.

Agriculture

Common Agricultural Policy (CAP) (EC, 2022)

The Common Agricultural Policy, launched in 1962, is a partnership among farmers, society, and agriculture. It consists of several regulating mechanisms of production, trade, and processing of agricultural products while promoting rural development. It aims farmers to take responsibility for the environment protection and sustainable agriculture, to look after biodiversity using wisely the natural resources, soil, air, and water. Moreover, it acts as a policy mechanism to keep the rural economy alive, promoting jobs in farming, agri-food industry and all the associated sectors.

The European Court of Auditors wrote a special report, back in 2021, on the Common Agricultural Policy and climate (ECA, 2021). The report looked to understand how the CAP was working to reduce the greenhouse gas emissions from agriculture, as more than a quarter of the budget was addressed to mitigation and adaptation to climate change. The result was that CAP climate action had a low impact on agricultural emissions and climate mitigation potential. In this way, the European Commission should increase their effort to reduce emissions from agriculture.

To reduce the impact of agricultural GHG and NH_3 emissions, this thesis evaluates feeding strategies to reduce NH_3 emissions from livestock manure, and processing manure to recover nutrients as a fertilizer reducing emissions and leakage during the production process.

Council Directive 91/676/EEC (Nitrates Directive)

The Nitrates Directive is one of the first EU legislation on the protection of waters against nitrates pollution caused by agriculture. It focuses on the reduction of water pollution from the use of fertilizers and livestock effluents by regulating the dosage of nitrogen that can be applied per hectare and year (i.e., 170 kg N/ha/y from livestock manure). It also includes application periods, manure storage facilities and buffer zones along water bodies to prevent lixiviation pollution (Council Directive 1991/676/EC).

This thesis connects with the Nitrates Directive by increasing the manure-N efficiency via reducing NH_3 emission and balancing the N input in livestock diets to their nutritional requirements (reducing N excretion in feces and urine). In this way leaching, deposition and further oxidation to nitrate can be reduced.

Regulation 2019/1009/EC to make fertilizing products available on the EU market (Fertilizing Product Regulation or FPR)

The FPR succeed the Fertilizers Regulation 2003/2003/EC establishing new rules to place fertilizers on the market, and replacing the labelling and packaging, reducing the existing trade barriers and potential risks for public safety from the use of certain fertilizers categories. It defines a variety of fertilizers besides the mineral ones (previous regulations), such as organic fertilizers. Thus, it opens new possibilities for new fertilizers production on a large scale, always meeting a certain quality and safety standards to achieve the CE label for free trade within the European Union markets.

The Regulation 2019/1009/EC set the minimum quality standards for the organic fertilizers in this thesis produced to boost circular economy and reduce the dependence on synthetic or mineral N, P, K. In this thesis, organic/bio-based fertilizers are produced trying to satisfy the quality and safety standards to achieve the CE label.

Real Decreto 1053/2022 de ordenación bovina

The Real Decreto 1053/2022 includes the basic rules for the zootechnical and health management of bovine cattle farms in relation to the maximum productive capacity depending on the type of cattle, and the minimum conditions of infrastructure, equipment and management, location, biosecurity, animal welfare and environmental requirements for manure management and emissions reduction.

In this thesis, nutritional strategies for emissions reduction are applied in an experimental dairy cow farm.

Waste

Directive 2008/98/EC (Waste Framework Directive)

The Waste Framework Directive encloses the collection, transport, recovery, and disposal of a wide variety of wastes. It indicates which measures should be taken to valorize them. It clearly separates waste and by-product, defining the valorization stage that a waste has to have to not be considered any more as a waste. A by-product is an “object or substance, resulting from a production process, the primary aim of which is not the production of that item” (Directive 2008/98/EC) that can be further used, while a waste has no further used in production, conversion or consumption.

Considering this Directive, this thesis is intended to treat livestock manure, waste, the proper way to get an organic fertilizer considered by-product to give a higher value to it, improving sustainability.

Emissions

REACH 2006/1907/EC – Registration, Evaluation, Authorization and Restriction of Chemicals.

"The purpose of this regulation is to ensure a high level of protection of human health and the environment" applied to community workplace and the environment. It aims to provide an efficient functioning of the internal market for substances and to achieve sustainable development, by eliminating or substituting dangerous substances. Manufacturers or importers of substances in quantity have to register their substances at ECHA (European Chemicals Agency). The amount of required information depends on the quantity (tons) of the substances.

Regarding the produced organic fertilizers, if at any point they have to be manufactured and/or imported, they would have to inform about their physical-chemical properties considered in the Annexes of the regulation.

Directive 2016/2284/EC or National Emission Ceilings Directive (NECD)

The National Emission Ceilings Directive sets the emission reduction of anthropogenic atmospheric emissions for the State Members of the EU. It is established that the pollutant emissions will be reported in the national programs for air pollution. As a part of this emission reduction, climate and agriculture areas are included for the reduction commitments for 2030.

In this thesis, N management and reduction is related to livestock feeding strategies, manure emission storage and recovery in the form of organic fertilizer.

Directive 2006/118/EC or Groundwater Directive

The Groundwater Directive establishes measures to control and prevent groundwater pollution, including (i) chemical status criteria, (ii) identification and reversal of significant and sustained upward trends and for the definition of starting points for trend reversals, (iii) complements the Directive 2000/60/EC in preventing or limiting inputs of pollutants into groundwater.

The Directive is directly linked with the Nitrates Directive aiming to prevent water pollution by nitrates from agriculture. Hence, it is important to take actions for better N management throughout the whole agronomic chain. The recovery and loss reduction of N is thus a principal factor in this thesis to avoid/reduce groundwater pollution.

Best Available Techniques (BAT) Reference Document for the Intensive Rearing of Poultry or Pigs

The BAT reference document entitled 'Intensive Rearing of Poultry or Pigs' forms part of a series presenting the results of an exchange of information between EU Member States, the industries concerned, non-governmental organizations promoting environmental protection, and the Commission, to draw up, review and, where necessary, update BAT reference documents as

required by Article 13(1) of the Directive 2010/75/EU on industrial emissions. This document is published by the European Commission pursuant to Article 13(6) of the Directive, and it covers nutritional management, feed preparation, housing, collection and storage of manure, processing of manure, manure land-spreading, and storage of dead animals.

Important issues for the implementation of Directive 2010/75/EU in the intensive rearing of poultry or pigs are ammonia emissions to air, total nitrogen and total phosphorus excreted. The reduction of ammonia emissions to air is an important topic in this thesis, and acidification, one of the proposed techniques, will be used to reduce them.

Circular Economy

Circular Economy Action Plan (CEAP)

The European Commission adopted the Circular Economy Action Plan in March 2020. It covers the entire life cycle of products, from the production to waste management and the market for secondary raw materials, and targets products design, encouraging sustainable consumption and waste generation, and local resources use as far as it is possible. It promotes recycling and re-use, bringing benefits not only to the environment but also to the economy. Moreover, it includes the will to develop an Integrated Nutrient Management Plan to ensure a more sustainable application of nutrients and the stimulation of the recovered nutrient market. This is an initiative that started in May 2022, and it is in preparation now: Nutrients – action plan for better management. This plan aims to evaluate nutrient pollution and inefficiencies in the nutrient cycle, to allow EU to define additional regulations and tools to improve food security, protect human health and preserve the ecosystem.

The present thesis contributes to the CEAP in the form of recovering nutrients from manure to convert them in organic fertilizers and reducing GHG and NH_3 emissions to protect human health and the environment.

Table 1. presented a summary of the defined legislations and the proposed approach in this thesis.

Table 1. Legislative framework and thesis working approach summary.

Category	Legislation	Objective	Thesis Approach
Agriculture	CAP	Support farmers, improve agricultural productivity, tackle climate change, maintain rural areas and rural economy	New sustainable techniques to support future farmers and agriculture, related to GHG and NH_3 emission reduction
	Nitrates Directive	Reduction of water pollution from the use of fertilizers and livestock effluents	Increase of manure-N efficiency via reducing NH_3 emission and balancing the N input in livestock diets to their nutritional requirements
	FPR	Rules to achieve the CE label for free trade of fertilizers within the European Union markets	Production of organic/bio-based fertilizers trying to satisfy the quality and safety standards to achieve the CE label through characterization and phytotoxicity tests
	Real Decreto 1053/2022	Rules for the zootechnical and health management of bovine cattle farms	Decrease of NH_3 emissions from manure via diet modifications
Waste	Waste Framework Directive	Establishes the differences between waste and by-product	Livestock manure treatments to produce high value fertilizers

Table 1. Continuation. Legislative framework and thesis working approach summary.

Emission	REACH	Ensure a high level of protection of human health and the environment	Full characterization of the final products (fertilizers) to prove their safety
	NECD	Sets national emission values for NOx, NMVOS, Sox, NH_3, and PM2.5	N management and reduction related to livestock feeding strategies, manure emission during storage and fertilizer production, and recovery in the form of organic fertilizer Collect local date through the evaluation of different strategies under Mediterranean climate
	Groundwater Directive	Measures to control and prevent groundwater pollution	Manure-N reduction, and emission control strategies to reduce the impact on NH_3 deposition, infiltration and lixiviation
	BAT	Strategies and techniques to improve agricultural practices	NH_3 emission reduction by using acidification of livestock manure, digestate or its derivates
Circular Economy	CEAP	Normalize sustainable products, ensure less waste, promote circular economy processes	As a circular agronomic process, the thesis intended to improve the use and recovery of nutrients from the animal production to the fertilizer production, taking into account the intermediate livestock manure management and storage while minimizing GHG and NH_3 emissions

References

Circular Economy Action Plan. https://environment.ec.europa.eu/strategy/circular-economy-action-plan_en. Accessed online October 2022.

Council Directive 91/676/EEC of 12 December 1991 concerning the protection of waters against pollution caused by nitrates from agricultural sources.

Directive 2006/118/EC of the European Parliament and of the Council of 12 December 2006 on the protection of groundwater against pollution and deterioration.

Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 on waste and repealing certain Directives.

Directive (EU) 2016/2284 of the European Parliament and of the Council of 14 December 2016 on the reduction of national emissions of certain atmospheric pollutants, amending Directive 2003/35/EC and repealing Directive 2001/81/EC.

European Commission. The common agricultural policy at a glance. https://agriculture.ec.europa.eu/common-agricultural-policy/cap-overview/cap-glance_en. Accessed online October 2022.

European Court of Auditors (ECA). 2021. Common Agricultural Policy and climate: half of EU climate spending but farm emissions are not decreasing. Special report No 16, 2021. Publications Office, LU.

Nutrients – action plan for better management. https://ec.europa.eu/info/law/better-regulation/have-your-say/initiatives/12899-Nutrients-action-plan-for-better-management_en. Accessed online October 2022.

Real Decreto 1053/2022, de 27 de diciembre, por el que se establecen normas básicas de ordenación de las granjas bovinas. BOE-A-2022-23053. https://www.boe.es/eli/es/rd/2022/12/27/1053.

Regulation (EU) 2018/848 of the European Parliament and of the Council of 30 May 2018 on organic production and labelling of organic products and repealing Council Regulation (EC) No 834/2007.

Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU 32eteorologi products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003.

Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of

Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC.

Santonja, G., Georgitzikis, K., Scalet, B., Montobbio, P., Roudier, S., Delgado, S.L. 2017. Best Available Techniques (BAT) Reference Document for the Intensive Rearing of Poultry or Pigs. Industrial Emissions Directive 2010/75/EU (Integrated Pollution Prevention and Control). EUR 28674 EN. Luxembourg (Luxembourg): Publications Office of the European Union; 2017. JRC107189

Chapter 4 – Locations, installations, and equipment description

This thesis has been developed in the framework of one of the 6 case studies of the H2020 project Circular Agronomics (773649), carried out in several locations in Catalonia, Spain. Besides, part of the experimentation has been performed at Ghent University, Belgium (Figure 6).

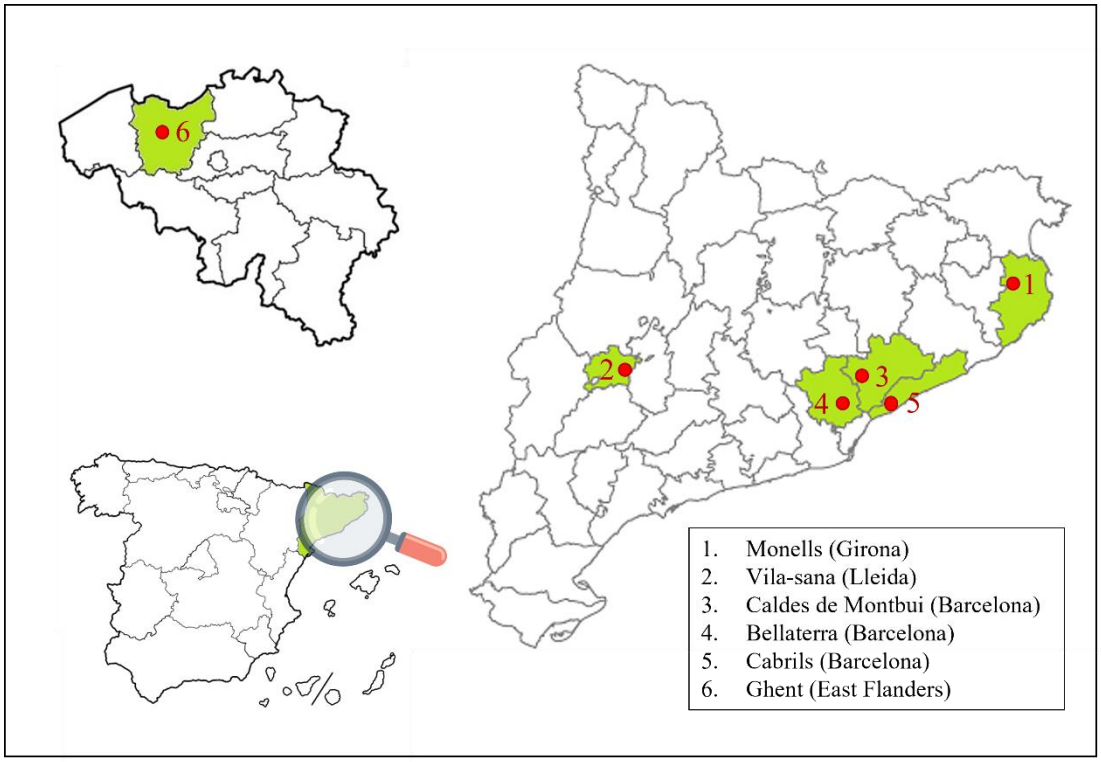


Figure 6. Location of the experimental set ups of the thesis.

1. EVAM: IRTA's experimental dairy farm

The EVAM research station of IRTA is located in Monells, Girona, Spain (Figure 7). It is a tool to serve the sector and the society, a research-experimentation platform capable to integrate knowledge about climatology, agronomy, vegetal production, water and livestock manure management, animal nutrition, welfare and management from a circularity point of view. One of their main activities is to evaluate management and nutritional strategies, because the facilities are equipped with tools that allow to record dairy cows individual feed intake in animals raised in groups, milk yield and its composition (fat and protein concentration), and BW. Besides, the personnel are capable to perform animal welfare evaluations, and to obtain samples to evaluate biochemical, immunological, hematological, microbial, and molecular biomarkers. Greenhouse gasses emissions (analyzed using a Lindvall hood) and organic residues (manure) management studies can also be conducted to evaluate mitigation strategies during manure management, such as acidification and storage tanks or covers.

Figure 7. EVAM satellite image (Google Maps, 2022).

EVAM, the dairy cow experimental farm, is an opened-barn designed to mechanically remove manure every hour using automated scrappers. The main area, for lactating dairy cows, has space for 120 animals distributed in 6 free stall pens with 20 cubicles (Figure 8a) and a milking parlor with a capacity for 20 cows that feed 2 milk cooling tanks of 4000 and 6000 liters.

The farm is also equipped with individual feeders in the milking parlor (Figure 8b) that allow to supplement concentrate feeds according to individual requirements estimated using individual cow performance data: milk yield, milk fat and protein composition, and BW, and TMR individual feed intake. The fact of adjusting individual nutritional needs using individual performance data is known as precision feeding system (Chapter 5).

Figure 8. EVAM. (a) Pens and (b) milking parlor.

2. Porgaporcs' biogas plant

The AD plant from Vila-sana, Catalunya, Spain, consists of 3 anaerobic reactors of 1,100 m^3 with a treatment capacity of 16500 m^3 per year of pig manure and agro-industrial residues (slaughterhouse sewage sludge, municipal sewage sludge, diary sewage sludge, and brewery sewage sludge) from the surrounding area. As a result, 750,000 – 800,000 Nm^3 of biogas are produced yearly and transformed to 2,250,000 – 2,500,000 heat kWh/year and 2,000,000 –

2,200,000 electric kWh/year. The digestate generated is mainly applied to fields directly as a fertilizer. However, with their participation in the Circular Agronomics H2020 and the development of this thesis, a semi-industrial pilot plant was constructed to produce improved organic fertilizers through greenhouse solar driers (Chapters 8 & 9). Figure 9 defines the treatment line that follows the digestate until it is dried and ready to use as organic fertilizer.

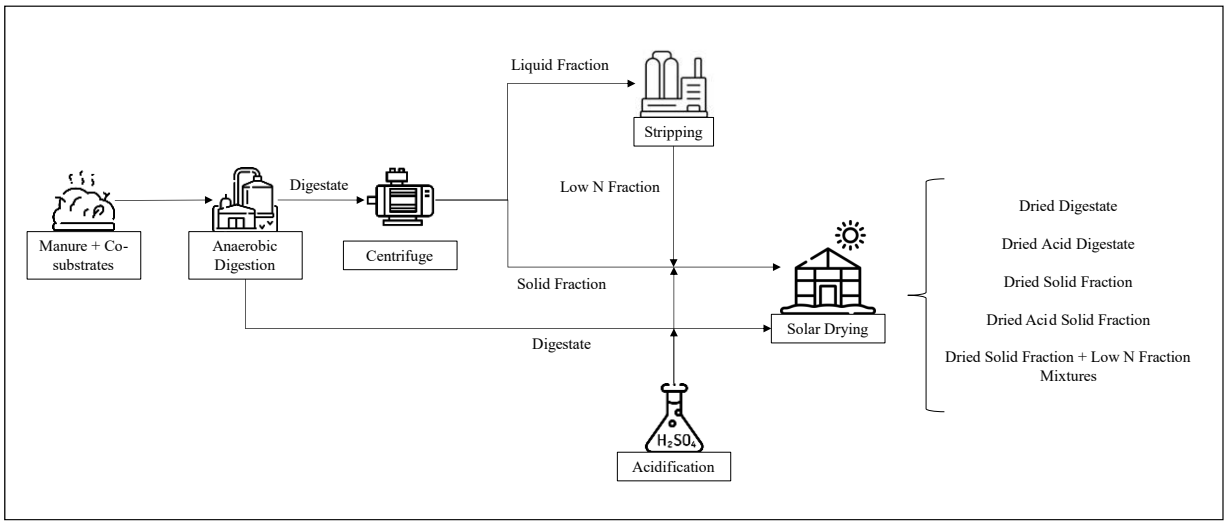


Figure 9. Digestate treatment line to produce dried organic fertilizer.

In each greenhouse solar drier, a turning machine designed by EMA depuració S.L. (Olot, Catalunya, Spain) (Figure 10) intended to homogenize the dried material and prevent the formation of a surface crust. Moreover, an acidification system was incorporated before the material was introduced to the greenhouses to decrease the pH and reduce NH_3 volatilization.

Figure 10. EMA depuració S.L. turning machine.

3. IRTA-Torre Marimon

Torre Marimon, located in Caldes de Montbui, Catalunya, Spain (Figure 11), is the headquarter of IRTA, a research institute in agri-food technologies. IRTA contributes to modernize, improve, boost competitiveness, and foster sustainable development in the sectors of agriculture, food, agroforestry, aquaculture, and fishing, as well as in all areas of activity directly or indirectly related to the supply of healthy, high-quality foodstuffs to end consumers, while also contributing to food safety and safe processing of foodstuffs and in general enhancing the health and well-being of the population.

Figure 11. IRTA Torre Marimon satellite image (Google Maps, 2022).

The analytical performance of all the samples and data collected during this thesis was done in this center. Moreover, storage experiments of bedding material from Montagi and EVAM (Chapters 5 & 6) were performed there. Figure 12a shows the sampling of gas emissions during the storage of precision feeding manure trial from EVAM. In addition, 4 pilot scale greenhouse solar dryers (Figure 12b) were designed to perform, on a smaller scale than in Vila-sana (Chapters 8 & 9), the drying of the solid fraction of digestate mixed with different ratios of the low nitrogen stream from a stripping process, aiming to reduce a waste stream from the biogas plant while improving the nutritional content of the resulting organic fertilizers. The pilot scale system was sensorized to control both temperature and humidity inside and outside the system.

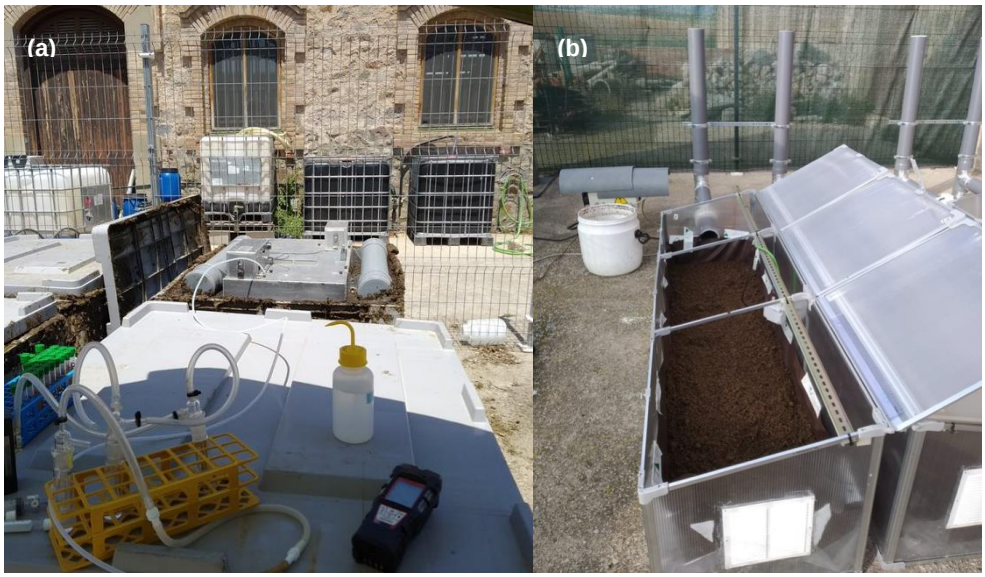


Figure 12. (a) Gas emission sampling during the storage of precision feeding manure and (b) Pilot scale greenhouse solar dryer.

4. Universitat Autònoma de Barcelona (UAB)

Universitat Autònoma de Barcelona (UAB) is a Catalan public university created in 1968. Most of its teaching centers and services are at the Bellaterra Campus (Vallès Occidental, Catalunya, Spain), located in a natural environment of 263 hectares of surface, of which 70% are forests and green areas. It also has teaching centers in Sabadell and the city of Barcelona. It has 13 faculties and schools, a hundred undergraduate degrees, 226 research groups and sixty research centers and institutes.

On one of this research group, "Grup de Microbiologia Molecular" (Molecular Microbiology), from the genetics and microbiology department, Chapter 6 was developed. It is a research group with more than 30 years of consolidated and recognized scientific trajectory, focused on:

DNA repair (SOS System) ad mechanisms of bacterial pathogenesis

Identification of bacteria targets for new antimicrobial compounds development

Bacteriophage therapy and phage biocontrol and bacterial phage-defense mechanisms

High-affinity iron uptake systems as targets for immunotherapy

In their laboratory ruminal fluid, from EVAM cows, was cultivated in a HAP selective media (Figure 13a) to isolate (Figure 13b), identify them (via 16S rDNA sequencing), and analyze their NH_3 production. Once the HAP were isolated, bacteriophages from the same ruminal fluid were concentrated and tested for affinity with them.

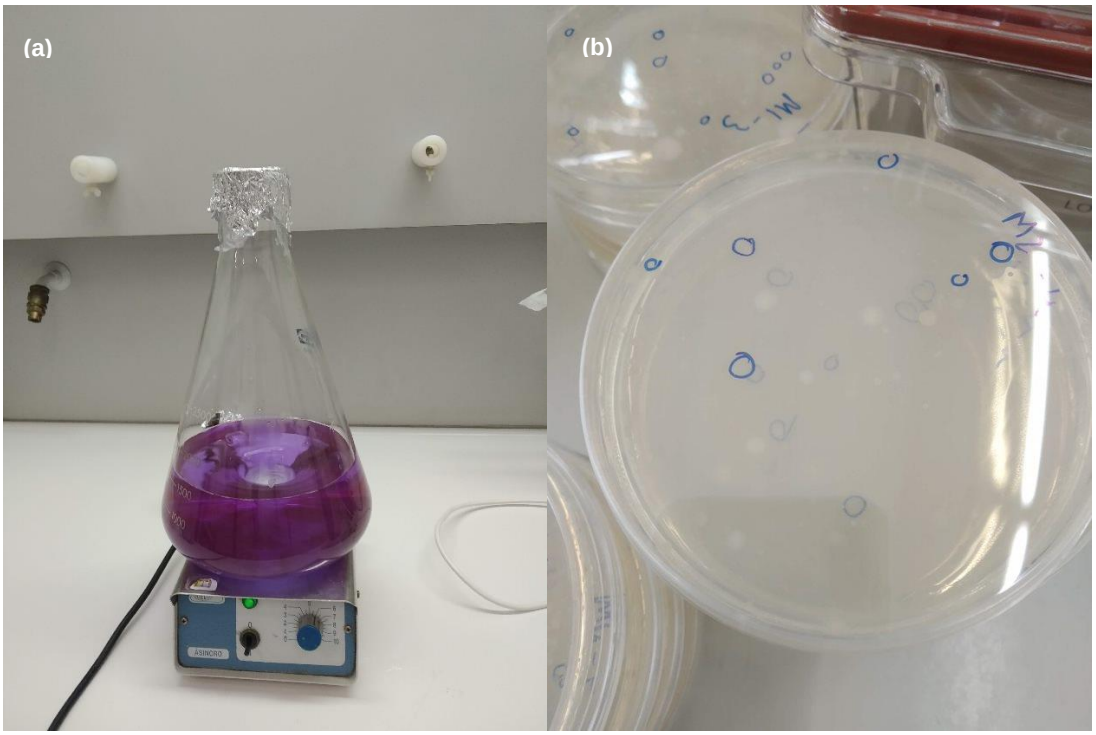


Figure 13. HAP specific culture media (a) and colonies isolation in cultivation plates (b).

5. IRTA Cabrils

IRTA Cabrils is located in Cabril, Catalunya, Spain. It is a research facility from IRTA focused on plant production, in a sustainable way, involving plagues and pathogens epidemiology, inoculation of beneficial microorganisms, genetic improvement, and organic waste management and treatment as compost. It has a surface of 62035 m^2 with greenhouses and fields where the experimental trials are performed (Figure 14 a & b).

Figure 14. IRTA Cabrils satellite image (Google Maps, 2023) (a). Greenhouse installation from Cabrils (b).

Germination assays (Figure 15a) with lettuce seeds from Chapter 9, and phytotoxicity trials (Figure 15b) with lettuce (var. Maravilla) from Chapters 8 and 9 were developed in the laboratory

and greenhouses from Cabrils to test the produced fertilizers. These experiments helped to determine the toxicity regarding nutrients and heavy metals absorption.

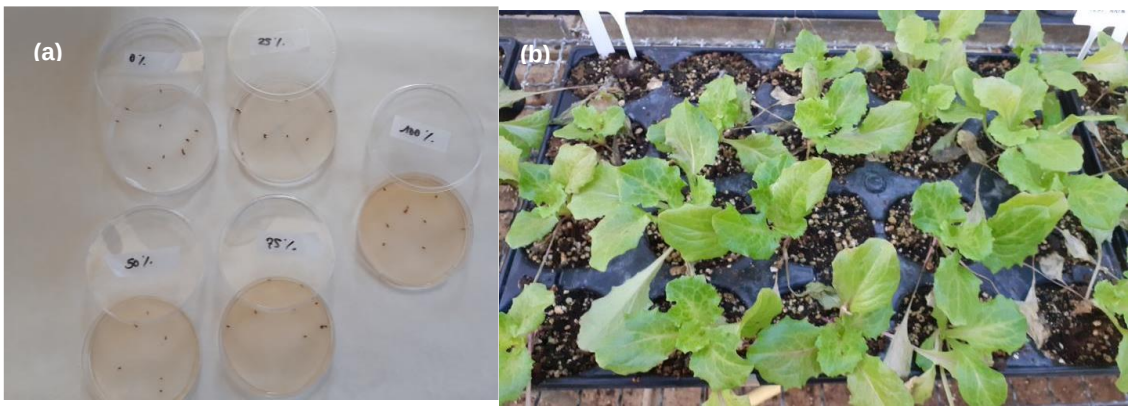


Figure 15. Germination (a) and pot trial assays (b) developed in Cabrils to test the phytotoxicity of the produced BBF.

6. Ghent University

Ghent University is a public research university located in Ghent, Belgium. It is ranked on the top 100 universities in the world and maintains international relationships across the world. It has more than 130 departments distributed around 11 faculties, offering more than 200 programs (including 64 Masters in English) and conducts in depth research within a wide range of scientific domains.

Plant and soil analytics of chapters 7 & 8 were performed there in the re-source lab (Figure 16a), from the green chemistry and technology department, at campus Coupure (Figure 16b). TN, TAN, TC, NO_3^-, TP, TK and heavy metals were analyzed to determine their behavior in the plant system as well as their accumulation in the edible part.

Figure 16. Re-source laboratory installations (a). Ghent University campus Coupure (b).

Chapter 5 – Effectiveness of precision feeding in reducing N excretion in dairy cattle

CRediT authorship contribution statement

Lluis Morey: Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft, Writing-Review & Editing. **Alex Bach:** Conceptualization, Methodology, Software, Writing-Review & Editing. **Daniel Sabrià:** Resources, Investigation. **Víctor Riau:** Methodology, Validation, Investigation, Writing – Original Draft, Writing-Review & Editing, Supervision, Project administration, Funding Acquisition. **Belén Fernández:** Writing – Writing-Review & Editing, Supervision. **Marta Terré:** Methodology, Validation, Formal analysis, Investigation, Writing-Review & Editing, Supervision, Project administration, Funding Acquisition.

Abstract

We hypothesized that precision feeding tools would allow to adjust nutrient requirements in grouped-fed cows, and consequently reduce N emissions. Two periods enrolling 56 and 58 dairy cows (average milk yield in period 1 = 36.2 ± 7.91 kg/d, period 2 = 32.4 ± 4.99 kg/d) were performed to evaluate nutrient adjustment of a precision feeding strategy. Animals were blocked by parity and distributed in four free-stall pens. Pens were randomly assigned to a conventional (CONV) or to a precision feeding scheme (PREC) for a 21-d period. The CONV group was offered a total mixed ration (TMR, 6.82 and 6.65 MJ/kg of dry matter, in period 1 and 2, respectively, and 165 g of crude protein (CP)/kg of dry matter in both periods; whereas PREC cows were fed a partial mixed ration (PMR) (6.65 and 6.40 MJ/kg of dry matter, 135 and 137 g of CP/kg of dry matter, in period 1 and 2, respectively) and a concentrate feed supplemented twice daily in the milking parlor, which contained different quantities of soybean meal, corn meal, and wheat middling's according to estimated nutritional needs of each cow above those supplied by the consumption of PMR. Individual daily nutritional needs and nutrients consumed from the PMR were calculated following NRC equations (2001) using a 10-d rolling average of performance data (milk yield and concentration of milk components, and BW). Daily PMR intake, milk yield and concentration of milk components were recorded in both periods. A N balance using urine and fecal spot sampling during the last 3 days of the study was performed in period 1, and stored manure gaseous emissions (NH_3, CH_4, N_2O, and CO_2) were measured for 2 wk in period 2. After 2 wk of adaptation to the diet, 82 cows homogeneously distributed in 4 days in milk (DIM) categories: early DIM (< 81), mid-early DIM (81 to 150), mid-late DIM (151 to 220), late DIM (> 220) were used to assess how energy and protein requirements were adjusted using either a CONV or a PREC feeding system. Dairy cows in both feeding systems were energetically overfed, and CONV cows tended to be more CP overfed in mid-late and late DIM cows than PREC fed cows. Total daily N urine excretion, and milk N urea concentration were greater in CONV than in PREC cows. There were no differences in NH_3 and N_2O emissions from the manure storage between PREC and CONV cows; however, CH_4 and CO_2 emissions from manure increased by 55 and 15%, respectively in PREC fed cows. Precision feeding system based on preceding average daily milk yield and composition can reduce N excretion without affecting short-term milking performance but increasing C gaseous emissions from manure. Further research is needed with long-term studies to corroborate performance results observed for 5 days.

The content of this chapter has been published as Morey, L., Bach, A., Sabrià, D., Riau, V., Fernández, B., Terré, M. 2023. Effectiveness of precision feeding in reducing N excretion in dairy cattle. Animal Feed Science and Technology. Available online 1 July 2023, 115722. https://doi.org/10.1016/j.anifeedsci.2023.115722.

Introduction

Excretion of N from livestock production contributes to environmental pollution. Losses of N from urine and manure as NH_3 can be oxidized to N_2O, one of the GHG that contributes to global warming and stratospheric ozone depletion (Hokestra *et al*., 2020; Zhang *et al*., 2020). Moreover, NH_3 can leach to the soil and groundwater leading to soil acidification (Zhang *et al*., 2020), nitrate (NO_3^-) accumulation in groundwater (Postigo *et al*., 2021), and NH_3 emission to the atmosphere. An important fraction of NH_3 emission is produced during manure storage (Kupper *et al*., 2020). At the top storage layer, where air and manure interface, NH_3 may disperse into the atmosphere depending, among other aspects, on manure temperature and air speed (VanderZaag *et al*., 2015).

Dairy cows need different amounts of protein depending on their milking performance and physiological status. However, under most commercial situations, cows are fed ad libitum in groups without being able to adjust individual nutrient supply. Typically, dairy rations for mid-lactation cows contain 165-170 g of CP/kg of dry matter (Groff, 2005; Olmos Colmenero and Broderick, 2006). As lactation advances, protein requirements decrease, and feeding 165 g of CP/kg of dry matter diets dairy to cows in late lactation may increase urinary and faecal N excretion and reduce profits (Law *et al*., 2009). Barros *et al*. (2017) reported that efficiency of N utilization (milk protein N yield /N intake; ENU) improved when feeding 144 g of CP/kg of dry matter diets to late-lactation cows, but lower CP levels had negative impacts on milk yield and efficiency.

Considering the potential environmental impact of feeding excessive amounts of CP to dairy cows along with the increasing demand for feed and food globally, there is an opportunity to adjust nutrient supply to cows to improve the efficiency of utilization of natural sources. Although cows are managed and fed in groups, each animal has its own requirements according to their stage of lactation, productivity, milk composition, and physiological state. Precision feeding strategies may allow matching nutrients supply with animal requirements to improve animal productivity while reducing environmental pollution and production costs (van Empel *et al*., 2016). Nowadays, the existence of several on-farm technologies that allow automatic collection of data such as concentrate intake, milk yield and composition, BW or animal behaviour may facilitate the application of precision feeding strategies. For example, Fisher *et al*. (2020) proposed to restrict feed intake to less efficient dairy cows using individual total mixed ration feeders, and several authors (Bach and Cabrera, 2017; Moore *et al*., 2020) have proposed to modulate the amount concentrate feed supplemented in AMS depending on milking performance of individual animals.

In a preliminary study, we observed that concentrate feed supplementation in the milking parlour according to milk yield and quality and mixed ration intake can reduce N urine excretion (Terré *et al*., 2020). Therefore, we hypothesized that feeding dairy cows a partial mixed ration (PMR) in

the feed bunk and a mix of concentrate feeds supplement in the milking parlour according to individual needs estimated based on individual 10-d rolling average of milking performance and individual PMR intake would optimize the use of dietary N and reduce manure NH_3 emissions. Thus, the objective of the present section was to evaluate the effects of a precision feeding strategy on N balance and gaseous emissions during manure storage, and how nutrients (protein and energy) were adjusted at different stages of lactation.

Materials and methods

Dairy cows were managed under the supervision of IRTA technicians and with the approval from the Animal Care Committee of the Government of Catalonia (authorization code 10640). Animals were housed at IRTA dairy farm (EVAM, Monells, Spain). The study consisted of two 21-day periods separated by 15 months (November 2019 and May 2021).

Animals and treatments

A total of 20 primiparous and 36 multiparous Holstein cows (729 ± 69.9 kg of BW; 36.2 ± 7.91 kg/d of milk; 155 ± 89.3 DIM; 2.1 ± 1.02 lactations, mean ± SD) in the first period, and a total of 29 primiparous and 29 multiparous Holstein dairy cows (708 ± 79.2 kg of BW; 32.4 ± 4.99 kg/d of milk; 160 ± 59.7 DIM; 2.0 ± 1.23 lactations, mean ± SD) in the second period with good initial health status were enrolled in a 21-day study (16 days of adaptation to the feeding system, and 5 days of measurements and nutrient calculations). Cows were blocked by parity (primiparous or multiparous) within feeding treatment and distributed in four free-stall pens (2 pens per feeding treatment). Animals were milked twice daily in a parallel milking parlour. Milk yield and fat and protein concentrations were recorded using electronic milk meters (AfiMilk, Afikim Ltd., Kibbutz Afikim, Israel) and an on-line system to determine milk components concentrations (AfiLab system, Afikim Ltd., Kibbutz Afikim, Israel). After each milking, all cows were weighed using AfiMilk SortWeight system (Afikim Ltd., Kibbutz, Israel). Each pen was equipped with 20 cubicles bedded with a mixture of compost and sawdust, 4 water troughs, and 15 electronic feed bins (MooFeeder, MooSystems, Cortes, Spain) to record individual feed intake. Animals in each of the 2 pens per period were fed a conventional (CONV) TMR (6.82 MJ/kg of dry matter, 165 g of CP/kg of DW in the first period; 6.65 MJ/kg of dry matter, 165 g of CP/kg of dry matter in the second period, (Table 2), and animals in the other 2 pens per period were fed under a precision feeding strategy (PREC). This consisted of feeding a PMR with lower energy and CP content than the CONV one (6.65 MJ/kg of dry matter, 135 g of CP/kg of dry matter in the first period; 6.40 MJ/kg of dry matter, 137 g of CP/kg of dry matter in the second period, Table 2) and a combination of 3 feeds (soybean, 458 g of CP, 136 g of NDF or neutral detergent fiber, 30.7 g of fat, 69.8 g of ash per kg of dry matter; a mix of 50% wheat and 50% corn, 101 g of CP, 126 g of NDF, 36.0 g of fat, 15.2 g of ashes per kg of dry matter; wheat middling's, 180 g of CP, 457 g of

NDF, 5.2 g of fat, 53.8 g of ash per kg of dry matter) in the milking parlour (supplement). These 3 ingredients were stored separately in 3 different silos that were connected to individual feeders at each milking place of the milking parlour. Automatically, on a daily basis, individual data were stored in a file that an algorithm, developed using Python (Van Rossum and Drake, 1995) as programming language, used to calculate, using NRC (2001) equations, the energy and CP needs for every individual cow using a 10-d rolling average of performance data recorded in our facilities (milk yield, milk fat and protein composition, and BW) from ten preceding days (estimated needs). Then, a 10-d rolling average daily consumption of nutrients from the PMR was computed considering individual dry matter intake and the estimated nutrient composition of the diet. Then, it was subtracted from the estimated needs to determine the amount of nutrients needed to be supplemented (if any) in the milking parlour for the PREC cows. The algorithm determined the amount of soybean, mix of wheat and corn, and wheat middling's to deliver in the milking parlour according to the nutrient composition of the feeds available, and the estimated needs above the precision PMR nutrient consumption. The algorithm consisted of linear programming optimization that found the optimum combination of the 3 ingredients complying with constraints on a minimum supply of energy, CP, and NDF within the limit of 3,000 g per day of concentrate fed in the parlour. Cows in PREC treatment not needing supplementation were offered 150 g of wheat middling's per milking to ensure that all cows had some feed offered while milking. The final amount of each ingredient was split in two feedings during each milking (at 0800 and 1900 h), meanwhile both, PMR and TMR were delivered in the feed bins twice daily while the animals were in the milking parlour.

Table 2. Ingredients and nutrient composition of the CONV and PREC partial mixed ration (PMR) fed in Studies 1 and 2. Abbreviations: NEL, Net energy for lactation; CP, crude protein; RDP, rumen degradable protein; RUP, rumen undegradable protein; Lys, lysine; Met, methionine; NFC, non fiber carbohydrates; NDF, neutral digestible fiber; ADF, acid detergent fiber.

	Period 1		Period 2	
Item	TMR	PMR	TMR	PMR
Ingredient (dry matter), kg				
Alfalfa hay	3.34	3.34		-
Rye grass silage	1.58	1.58		-
Rye grass hay	1.74	1.74		-
Barley silage	3.25	3.25		-
Barley straw	0.74	0.74		-
Wheat silage	-	-	3.76	3.76
Corn silage	-	-	3.70	3.70
Alfalfa silage	-	-	3.49	3.49
Oat hay	-	-	1.75	1.75
Corn meal	7.93	7.49	5.27	4.84
Wheat meal	0.45	-	1.41	0.97
Canola meal	1.81	1.81	-	-
Soybean meal	2.34	0.55	2.75	1.00
Soybean hulls	-	-	1.17	1.17
Wheat middlings	0.89	0.45	0.82	0.82
Hydrogenated vegetable fat	0.42	0.42	-	-
Calcium carbonate	0.08	0.08	0.14	0.14
Magnesium oxide	0.03	0.03	0.04	0.04
Salt	0.08	0.08	0.05	0.05
Vitamin minerals premix [2]	0.04	0.04	0.05	0.05

Table 2. Continuation. Ingredients and nutrient composition of the CONV and PREC partial mixed ration (PMR) fed in Studies 1 and 2. Abbreviations: NEL, Net energy for lactation; CP, crude protein; RDP, rumen degradable protein; RUP, rumen undegradable protein; Lys, lysine; Met, methionine; NFC, non fiber carbohydrates; NDF, neutral digestible fiber; ADF, acid detergent fiber.

	Period 1		Period 2	
Item	TMR	PMR	TMR	PMR
Nutrient composition				
Dry Matter, %	60.5	60.7	53.4	53.1
NEL, Mcal/kg of DW [3]	1.63	1.59	1.59	1.53
CP, % of DW	16.4	13.9	16.5	13.7
RDP, % of DW [3]	10.7	8.8	10.8	8.9
RUP, % of DW [3]	5.7	5.1	5.7	4.8
Metabolizable protein, % of dry matter [3]	10.9	9.3	11.1	9.2
Digestible Lys, % of MP [3]	5.33	4.53	4.98	4.13
Digestible Met, % of MP [3]	2.04	1.74	2.01	1.67
NFC, % of dry matter [4]	33.4	31.5	41.9	41.5
NDF, % of dry matter	38.9	43.1	32.2	35.0
ADF, % of dry matter	23.4	21.8	19.8	21.7
Fat, % of dry matter	4.7	4.9	3.0	3.1
Ash, % of dry matter	6.6	6.6	6.4	6.6

[1] TMR: total mixed ration offered to conventional fed cows; PMR: partial mixed ration offered to precision feeding fed cows

[2] 14.6% Ca, 0.03% Na, 4.48% Mg, 2,250,000 UI/kg Vitamin A, 8,8000 mg/kg Vitamin E, 665,000 UI/kg Vitamin D3, 40 mg/kg Co, 30,000 mg/kg Zn, 150 mg/kg Se, 20,000 mg/kg Fe, 250 mg/kg I, 30,000 mg/kg Mn, 5,000 mg/kg Cu, 1,500 mg/kg Butylhydroxytoluene, 279,949 mg/kg sepiolite

[3] Estimated using NRC (2001)

[4] Calculated 100 – (CP+NDF+EE+ash)

Sampling and Measurements

Individual feed intake was monitored daily using MooFeeders (MooSystems, Cortes, Spain). Both PMR and feed ingredients composing the concentrate offered in the parlour were sampled weekly and composited for the 3-wk period for subsequent nutrient composition analysis. Samples were frozen at -20ºC to subsequently determine dry matter (method 934.01), N (method 984.13), ether extract (method 920.39), and ash (method 942.05) following AOAC International (2000) and for aNDF according to Van Soest *et al*. (1991) using sodium sulphite and heat-stable amylase. Non-fibre carbohydrates were calculated as 100 minus CP, aNDF, fat, and ash (NRC, 2001). For all animals involved in the study, energy-corrected milk (ECM) was calculated using the equation

(Bernard, 1997): (0.3246 × milk) + (12.86 × kg fat) + (7.04 × kg protein) using AfiLab system data, and feed efficiency was computed as the of ratio of ECM to total dry matter intake. Furthermore, milk samples of all animals enrolled in the study were obtained at day 19 of study in tubes containing 2-bromo-2-nitropropane-1,3-diol, which were analysed for urea, using infrared spectroscopy (MilkoScan™ 7; Foss Iberia S.A., Barcelona, Spain).

From the first group of animals, a set of 26 cows homogeneously distributed by LN (Lactation number) and DIM in both treatments were selected to perform a N balance. Faecal spot samples (about 300 g) from the rectum and urine spot samples (about 250 mL) obtained by massaging the perivaginal area were collected from d 19 to 21 of study, at different times: on day 1 at 0800, 1400, 2000, 0200 h; on day 2 at 1000, 1600, 2200, 0400, and on day 3 at 1200, 1800, 0000, 0600 h. On these 3 consecutive days, samples of both PMR offers were collected and composited for further nutrient composition analysis. Faecal samples from the dairy cows were dried at 60ºC for 4 d, ground at 1 mm, and further composited on a dry weight basis by cow. Composite samples were analysed for dry matter, total N (TN) content (Standard methods for the Examination of Water and Wastewater, APHA 2005 using Kjeldahl digester and distillation unit from Buchi®, Switzerland), 50et, and acid insoluble ash (AIA), which was used as an internal marker to estimate faecal output (Sales and Janssens, 2003). Samples of both PMR were composited and analysed for dry matter, CP, aNDF, and AIA. Total tract apparent digestibility was calculated based on AIA concentration in the rations and faeces. Aliquots of 30 mL of urine were immediately acidified with 0.1M sulfuric acid, diluted 1:5.7, and stored at -20ºC. These samples were composited by cow and analysed for TN (APHA 2005, using Kjeldahl digester and distillation unit from Buchi®, Switzerland). A 15-mL aliquot was used to determine urine creatinine (Jaffe Method using Olympus System Reagent®, Beckman Coulter®, Ireland). Daily urine excretion was estimated assuming a urinary creatinine excretion of 29 mg/kg of BW/d (Valadares *et al*., 1999), and then, combined with urine N concentrations, used to estimate total urinary N excretion. Efficiency of N utilization was calculated as the proportion of N secreted in milk relative to total N intake. Furthermore, milk samples from the 26 cows selected for the N balance were collected in tubes containing 2-bromo-2-nitropropane-1,3-diol on days 19, 20, and 21 of study and composited for the morning and afternoon milking according to milk weights and analysed for fat, lactose, TS, non-fat, urea, and somatic cell count (SCC) using infrared spectroscopy as previously described. Lastly, blood samples (10 mL) were collected in ethylene diamine tetra acetic tubes (Vacutainer, Becton Dickinson, Madrid Spain) from the coccygeal vessels 5 h after the morning feeding on d 21. They were centrifuged at 1,500 x g for 10 min and frozen at -20 ºC for further plasma urea determination following the L-glutamate dehydrogenase method (Talke and Schubert, 1965) (Olympus System Reagent®, Beckman Coulter®, Irlanda). Change in BW, in

these cows, was calculated as the difference between average BW the week before the study begun and the last week of study.

Lastly, the last week of the study, manure scrapers were stopped overnight to accumulate manure in the pens. Next morning, the scrappers were activated to bring all the manure accumulated at the end of the alley to collect two plastic boxes of 0.675 m^3 (600 L capacity; internal dimensions: 91x111x62 cm) each per treatment to simulate manure ponds. Boxes were manually filled to the top with 0.6 m^3 of manure directly from the pen floor (the manure sample for the CONV was a mixture of the two pens in CONV, and the manure sample for PREC was a mixture of the manure from the two pens in PREC), and they were kept at ambient temperature. Then, on a weekly basis, the evolution of manure composition and GHG and NH_3 emissions were monitored during manure storage for 14 days.

Manure samples were collected weekly and analysed following the Standard methods (APHA, 2005) to determine DM content (2540G), total ammonia N (TAN) (4500-NH3-C) and pH, directly measured in homogenized samples using a pH-meter (4500-H+) (pH basic 20, Crison, Spain). Total carbon (TC) and TN were determined by using an elemental analyzer LECO® (Leco Corporation, Michigan, USA) (ISO 13878: Soil Quality – Determination of Total Nitrogen Content by Dry Combustion). Gaseous emissions were collected weekly using a dynamic chamber coupled to an NH_3 sensor Riken Keiki GX-6000 (RKI Analytical Instruments GmbH, Bad Homburg vor der Höhe, Germany) for NH_3 emissions. Vacuum tubes, Labco Exetainer® (Labco Limited, Lampeter, United Kingdom) were used to collect 30 mL of gas for the GHG emissions. CH_4, CO_2 and N_2O were analysed as described in Torrellas *et al*. (2018), sealing the area between the surface and the air, avoiding gas losses, and creating a controlled airflow of 0.2-0.3 $m \cdot s^{-1}$ following EPA recommendations (EPA, 2001). Emission fluxes ($mg \cdot m^{-2} \cdot h^{-1}$) at 25ºC of NH_3 and GHG were calculated by multiplying the gas concentration (mg/m^3) by the dynamic chamber internal flux (30 $m^3 \cdot m^{-2} \cdot h^{-1}$), the flow ($m^3 \cdot m^{-2} \cdot h^{-1}$) emitted per unit area of the chamber (m^2). The total gas emission after 14 d was calculated by the trapezoidal method of integration (Levy *et al*., 2017; Dalby *et al*., 2022).

Calculations

To assess how cows in CONV and PREC adjusted daily to their energy and protein requirements according to their stage of lactation, cows were classified in 4 DIM categories: early DIM (< 81), mid-early DIM (81 to 150), mid-late DIM (151 to 220), late DIM (> 220). Then, 82 animals (156 ± 66.0 DIM, 2.0 ± 1.19 lactations, 34.0 ± 6.13 kg/d, 707 ± 69.5 kg of BW) from both treatments homogeneously distributed within DIM category and period were selected to evaluate the algorithm for precision feeding by calculating total energy and protein intake for the last 5 d of

the study, and the proportion of nutrients consumed above or below their estimated requirements for their production level.

Statistical analysis

All data were analysed using the SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA). Comparisons with $P < 0.05$ were considered significant, whereas comparisons with $0.05 \leq P < 0.10$ were presented as tendencies. The experimental unit for the statistical analysis was cow because treatments were applied at the cow level. Performance and nutrient evaluation (nutrient requirements, TMR and PMR nutrient intake, and proportion of nutrients overconsumed) data were analysed with a mixed-effects model that included the fixed effects of feeding system, lactation stage (early, mid-early, mid-late, and late DIM), day of study (from 17 to 21), and their 2-way and 3-way interactions plus the random effect of pen, study, and cow within pen (to account for the dependence of animals within pen), parity (primiparous or multiparous) as a block. Day entered in the model as a repeated measure using an autoregressive order 1 or compound symmetry variance-covariance matrix according to the lowest Bayesian information criterion. The 3-way interaction was not significant for any parameter, and it was removed from the final model. The model used was:

$$Y_{ijklmno} = \mu + C_i\,(P_j) + P_j + S_k + L_l + T_m + M_n + D_o + (T_m \times M_n) + (M_n \times D_o) + (T_m \times D_o) + (T_m \times M_n \times D_o) + e_{ijklmno},$$

where Y_{ijklmn} is the dependent variable, μ is the overall mean, $C_i\,(P_j)$ is the cow within pen, P_j is the pen, S_k is the study, L_l is the lactation number, T_m is the treatment (CONV or PREC), M_n is the categorical DIM, D_o (k = 17 – 21) is the day of study, $(T_m \times M_n)$ is the interaction between treatment and categorical DIM, $(M_n \times D_o)$ is the interaction between categorical DIM and day, $(T_m \times D_o)$ is the interaction between treatment and day, $(T_m \times M_n \times D_o)$ is the interaction between treatment, categorical DIM and day, and e_{ijklmn} is the residual error. Pen, study, and cow within pen were random effects, lactation number a block, and the other parameters were fixed effects.

Data pertaining to the N balance in Period 1, were analysed with an analysis of variance considering the feeding system as fixed effects, parity as a block, and pen as a random effect. The model used was:

$$Y_{ijklm} = \mu + P_i + L_j + T_k + e_{ijk},$$

where Y_{ijklm} is the dependent variable, μ is the overall mean, P_i is the pen, L_j is the lactation number, T_k is the treatment (CONV or PREC), and e_{ijk} is the residual error. Pen was the random effect, lactation number a block, and feeding treatment was fixed effects.

Gaseous emissions from boxes in Period 2 were analysed with a mixed-effects model that accounted for the fixed effects of feeding system, day of sampling, and their interaction plus the

random effect of the box. Day entered the model as a repeated measure using the autoregressive order 1 or compound symmetry variance-covariance matrix according to the lowest Bayesian information criterion. Herein, the experimental unit was box (n = 2).

The model used was:

$Y_{ijk} = \mu + B_i + T_j + D_k + (T_j \times D_k) + e_{ijk}$,

where Y_{ijk} is the dependent variable, μ is the overall mean, B_i is the box, T_j is the treatment (CONV or PREC), D_k is the day of sampling, $T_j \times D_k$ is the interaction between treatment and the day of sampling, and e_{ijk} is the residual error. Box was a random effect, and the other parameters were fixed effects.

Results

From the initial dataset of 114 animals, a group of 26 cows from period 1 was selected to perform a N balance, another group of 82 cows from both periods were selected for the evaluation of nutrient adjustment and performance responses. All 58 animas from the second period were considered for the evaluation of manure emissions.

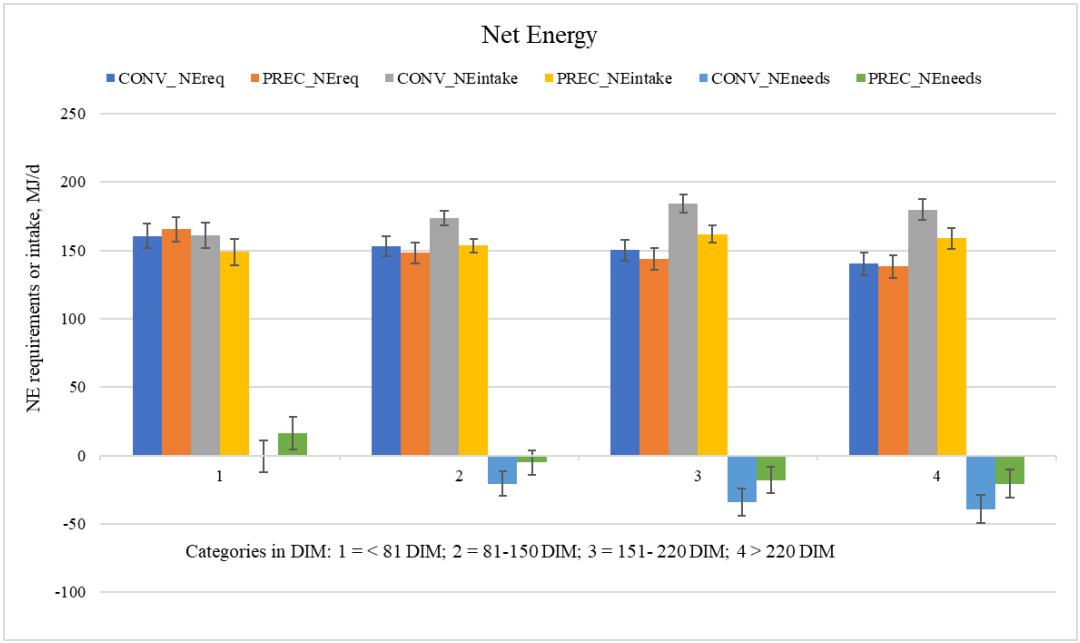
Diets

Ingredient composition of the diets was different in both periods because they were conducted in different seasons (Table 3), and forage availability was different. Dietary dry matter, aNDF, ADF, and fat concentrations were slightly greater in period 1 than in period 2, but CP content was kept similar in both studies.

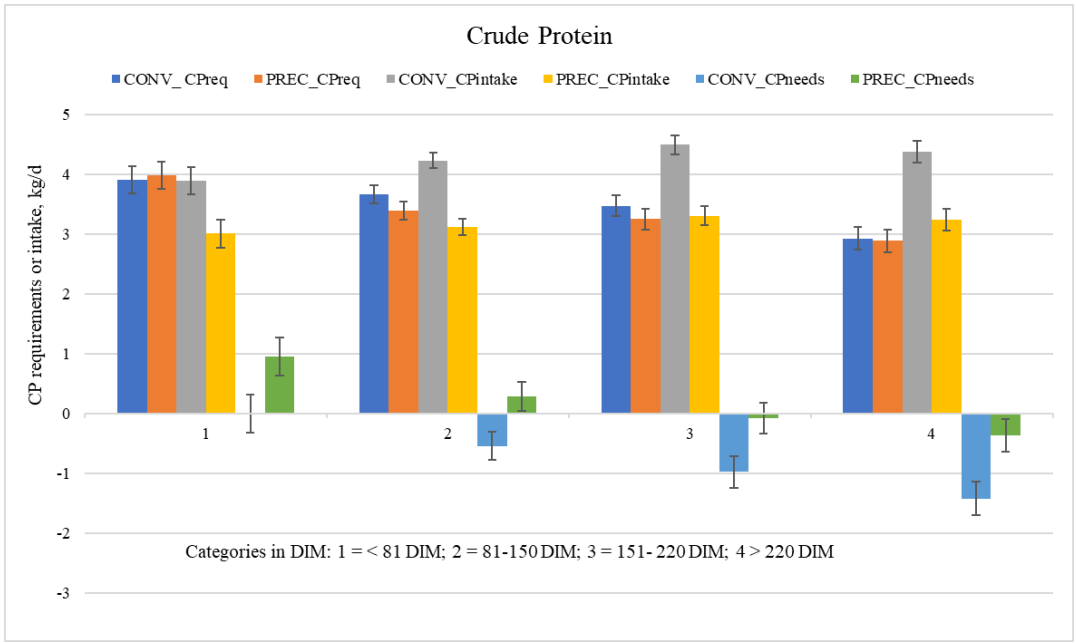
Nutrient adjustment and performance

Estimated energy (Figure 17a) and protein (Figure 17b) requirements decreased ($P<0.001$) with DIM categories and increased with day of study, and there were slightly differences ($P<0.05$) between days and treatments. Estimated energy requirements increased in CONV cows from 20 to 21 d, and estimated CP requirements increased from 17 to 18 d, and from 20 to 21 d of study; whereas estimated energy requirements PREC fed cows were similar during the last 5 d of study, but estimated CP requirements increased with time. Neither energy nor CP estimated requirements differed between treatments within DIM categories. As expected, energy and CP intake (Figure 17a & 17b) from the TMR and PMR was greater ($P<0.001$) in CONV than in PREC cows and there were slight differences ($P<0.001$) in the daily variation between treatments and DIM categories. Cows in both treatments and DIM categories covered their estimated energy and CP requirements solely with consumption of TMR and PMR, except for PREC cows in the early DIM category that required a supplement of 16.4 extra MJ of Nel/d, and 955 g of CP/d, and PREC cows in mid-early DIM category that needed 287 extra g of CP/d (Figure 17a & 17b). Both CONV

and PREC cows overconsumed energy without differences between DIM categories and treatments (Figure 18), and with slight variations (P<0.05) between days of study. However, CONV cows consumed more (P<0.05) CP above their requirements than that consumed by PREC cows, and these differences tended (P=0.09) to be greater in cows with mid-late and late DIM (Figure 18).



a)



b)

Figure 17. Least square means of NE (a) and CP (b) requirements, intake and needs of dairy cows fed under a conventional or a precision feeding system using performance data of the previous 10 rolling days and NRC (2001) equations, and categorized in 4 DIM categories (data from periods 1 and 2).

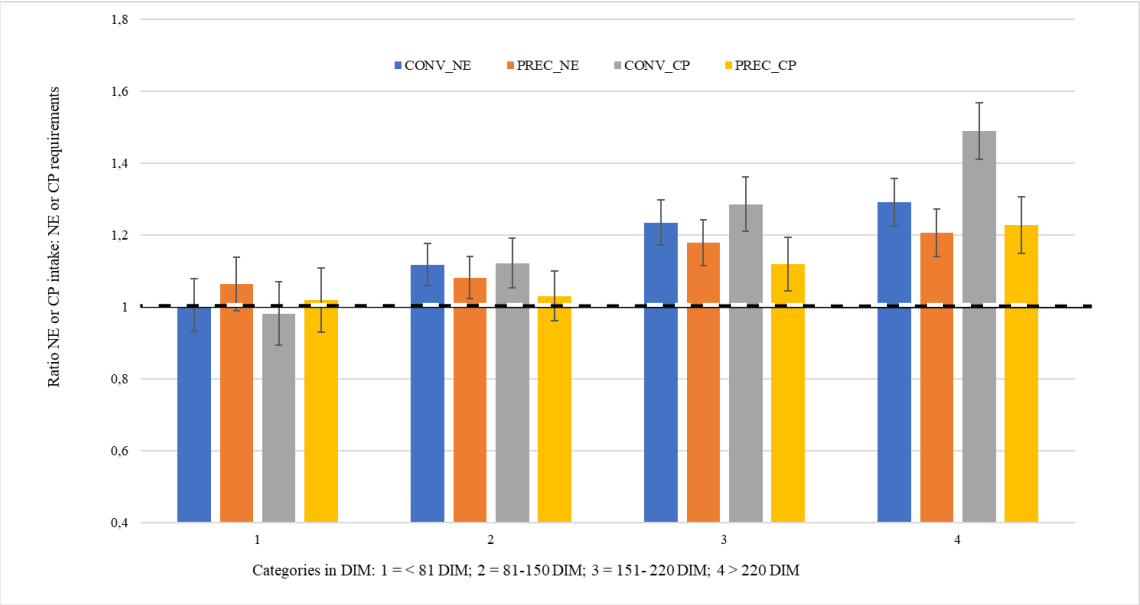


Figure 18. Least square means of the proportion of energy and CP overfed in cows fed under a conventional or a precision feeding system using performance data of the previous 10 rolling days and NRC (2001) equations and categorized in 4 DIM categories (data from periods 1 and 2). Values higher than 1 are overestimated and under 1 are underestimated.

Although it was not the main objective herein, due to the short duration of the study, cows produced similar amounts of milk in both feeding treatments, and as expected it progressively decreased ($p < 0.01$) as DIM category increased. Milk components (fat and protein) did not differ between feeding treatments, and both increased ($p < 0.01$) by DIM category. Milk fat content in PREC cows tended ($p = 0.09$) to be similar whereas in CONV fed cows it tended to increase for mid-early and mid-late DIM (Table 3). On the other hand, milk N urea was lower ($p < 0.05$) in PREC than in CONV cows from early-mid DIM to late DIM, whereas it did not differ in cows with early DIM between feeding treatments. Total dry matter intake was similar in both feeding schemes, but CP intake was lower ($p < 0.05$) in PREC cows with more than 81 DIM in comparison with CONV cows (Table 2). Dietary energy density of the total diet, considering feed supplements in the milking parlour, was lower ($p < 0.001$) and aNDF content greater ($p < 0.001$) in PREC than in CONV, but it did not differ across DIM categories within treatment. However, dietary CP content decreased ($p < 0.05$) within DIM category in PREC and it was constant in CONV.

Table 3. Least square means of milk yield and composition of dairy cows fed under a conventional (CONV) or a precision (PREC) feeding system during a period of 5 days, after 16 days of adaptation (period 1 and 2).

	Treatments			p-values		
	CONV	PREC	SEM	FS	SL	FSxSL
Cows, n	41	41	-	-	-	-
Body weight, kg	713	708	12.5	0.76	<0.001	0.95
Total dry matter intake, kg/d	26.0	25.7	0.64	0.71	0.10	0.19
Total CP intake, kg/d	4.25	3.72	0.132	0.002	0.15	0.03
Diet CP, g/kg of DM	164	145	1.4	<0.001	0.007	0.018
Diet aNDF, g/kg of DM	356	379	35.8	<0.001	0.05	0.31
Diet NEl, MJ/kg DM	6.74	6.57	0.100	<0.001	0.21	0.12
Milk yield, kg/d	34.0	33.8	1.70	0.90	<0.001	0.62
ECM, kg/d	35.4	35.4	2.61	0.98	<0.001	0.53
Milk fat, %	3.77	3.81	0.254	0.59	<0.001	0.09
Milk protein, %	3.43	3.39	0.044	0.34	<0.001	0.97
Milk N urea, mg/dL	10.8	6.8	25.05	<0.001	0.73	0.02
Feed efficiency[5]	1.41	1.40	0.129	0.84	<0.001	0.70

[1] FS = effect of the feeding system; SL = effect of stage of lactation; FSxSL = effect of the interaction of feeding treatment with stage of lactation

[2] Standard error of the mean

[3] Milk fat and protein component concentrations daily measured using the AfiLab system

[4] Energy corrected milk calculated as (0.3246 × milk) + (12.86 × kg fat) + (7.04 × kg protein) (Bernard, 1997)

[5] Feed efficiency calculated as (Energy corrected milk) / (Total dry matter intake)

Nitrogen balance and diet digestibility

During the 3 d of N balance duration, N intake and N concentration in milk and faeces were similar in both feeding regimes, but N in urine was greater ($p < 0.05$) in CONV than in PREC

cows (Table 4). Digestibility of dry matter and apparent CP digestibility were not affected by feeding regime, but aNDF digestibility tended to be greater ($p = 0.08$) in PREC than in CONV cows. Lastly, plasma urea concentration was unaffected by feeding strategy.

Table 4. Least square means of digestibility and nitrogen balance of dairy cows fed under a conventional (CONV) or a precision (PREC) feeding system (days 19 to 21 of period 1).

	Treatments			
	CONV	PREC	SEM[2]	P-value[1]
Cows, n	13	13		FS
Apparent digestibility, g/100 g				
Dry Matter	60.5	61.8	1.07	0.42
NDF	42.6	46.9	1.72	0.08
CP	48.1	45.0	1.34	0.12
N balance,				
N intake, g/d	636	636	56.1	0.99
Milk protein N, g/d	189	182	11.5	0.64
Urinary total N, g/d	196	140	12.8	0.006
Faecal N, g/d	305	308	19.9	0.91
N retained, g/d	-51.4	-0.4	35.1	0.32
BW change, kg	-12	-3	7.3	0.35
As a proportion of N intake, g/100 g				
Milk N	29.8	29.5	1.24	0.85
Urine N	31.2	22.6	3.20	0.07
Faecal N	48.4	49.8	1.35	0.46
Plasma urea, mg/dL	18.2	15.5	1.45	0.19

[1] FS = effect of the feeding system

[2] Standard error of the mean

Manure emissions

Manure of PREC cows had lower ($p < 0.05$) pH and $N\text{-}NH_4^+$ concentration than manure from CONV cows. Although TC and ADF concentration were similar in both treatments, the ratio C to N tended to be greater ($p < 0.10$) in the manure from PREC than in that from CONV cows (Table 3). NH_3 emissions decreased throughout sampling days, and although they did not differ between both feeding systems (Table 5), they were numerically reduced by 20% in PREC in comparison to CONV manure. In contrast, CO_2 and CH_4 emissions increased ($p < 0.05$) in PREC manure, by 14% for CO_2 and 55% for CH_4 compared with CONV manure (Table 5). The emissions of CO_2 and CH_4 emissions from manure in PREC increased throughout the 14 d of monitoring, in contrast to CONV manure, which CO_2 and CH_4 emissions started to decrease by 14 d (Figure 19). N_2O emissions tended ($p = 0.06$) to be lower in PREC compared with CONV manure at 14 d of sampling (11%).

Table 5. Least square means of manure characterization and gas emission production (mg/m^2 h) under conventional (CONV) and precision (PREC) feeding treatments for 14 days using manure from period 2.

	Feeding system [1]			p-values [2]		
	CONV	PREC	SEM [3]	FS	time	Fsxtime
Number of boxes	2	2				
Manure characterization						
pH	7.45	7.17	0.045	0.01	0.05	0.02
Conductivity	4.07	3.23	0.882	0.54	0.06	0.71
Total Solid content (%)	13.6	13.7	0.65	0.89	0.03	0.89
Volatile Solid content (%)	10.7	10.8	0.46	0.85	0.03	0.89
Total NH3, mg/kg [4]	13,385	10,239	458.77	0.005	0.002	0.55
Total N, % [4]	4.53	3.87	0.14	0.08	0.002	0.008
Total C, % [4]	50.5	47.6	2.22	0.45	0.77	0.42
Ratio C/N	11.2	12.3	0.28	0.10	0.16	0.19
NDF, % [4]	51.4	41.3	3.73	0.13	0.17	0.76
ADF, % [4]	37.4	33.9	2.12	0.30	0.81	0.74
Gas emissions, mg/m^{2}h						
NH_3	725.6	510.0	111.75	0.24	0.006	0.85
CO_2	16,868	26,799	1,918.1	0.02	0.004	0.010
CH_4	1586	3941	512.5	0.03	0.015	0.041
N_2O	28.1	24.4	1.21	0.10	<0.001	0.06

[1] CONV = cows fed a TMR in the feed bin scales; PREC = cows fed a PMR plus a concentrate feed supplementation in the milking parlor

[2] FS = effect of the feeding system; time = effect of day of study; Fsxtime = effect of the interaction between feeding system with day of study

[3] Standard error of the mean

[4] Dry weight

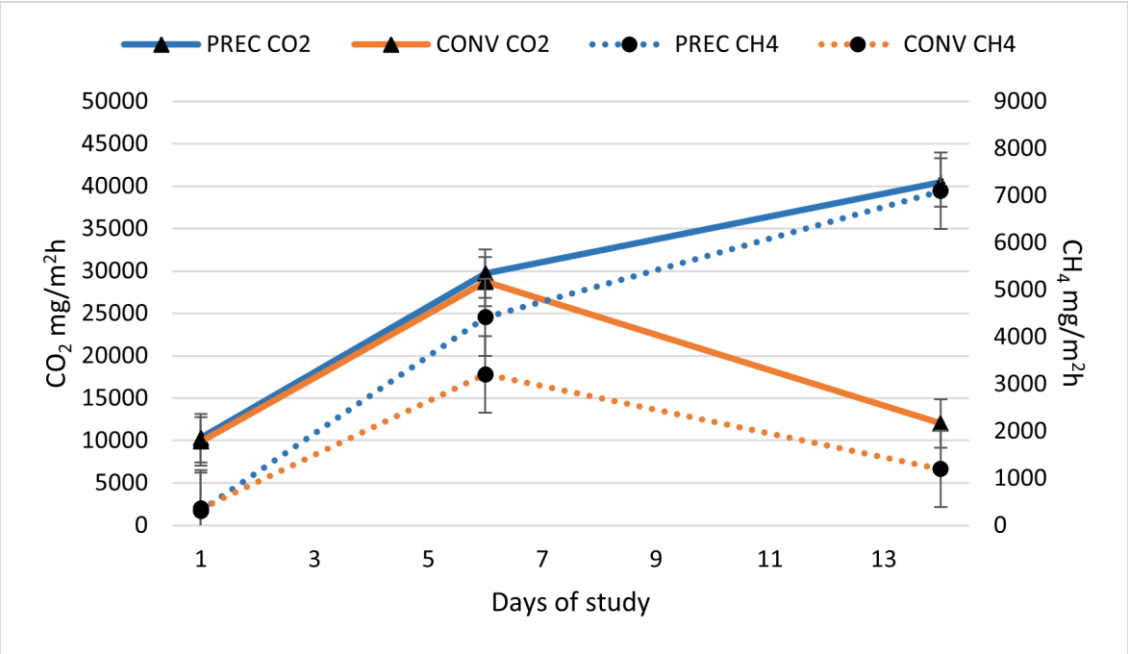


Figure 19. Least square means and standard error of the mean of the evolution of CH_4 (right axis) and CO_2 (left axis) emissions from manure of cows fed under a conventional (CONV) or a precision (PREC) feeding system during 2-week storage (period 2).

Discussion

Differences observed in milk components in the different lactation stage categories such as an increase in fat, protein in greater DIM cows are widely described in the literature (Linn, 1988), and the main interest in splitting data herein in DIM categories was to determine whether a precision feeding system would have the same capacity to modulate nutrient supply according to stage of lactation and thus elicit a similar response in performance throughout all phases of lactation.

Cows fed in CONV feeding system overconsumed CP except those with less than 81 DIM. However, PREC cows consumed the exact of CP needed to satisfy their estimated requirements in cows <150 DIM, but PREC cows with >150 DIM overconsumed CP. Cows in CONV received a fixed CP concentration in their diet, whereas PREC cows were offered a range between 133 and 167 g of CP/kg of dry matter. In general, PREC cows consumed less CP than CONV cows, but PREC and CONV cows used in the N balance had similar N intake, probably because the selected cows for N balance averaged 161 DIM. Olmos Colmenero and Broderick (2006) evaluated different dietary CP levels (from 135 to 194 g of CP/kg of DM) and reported no changes in production traits, but they also reported a decrease in milk urea N (MUN) as CP levels in the diet decreased. However, Law *et al*. (2009) described performance benefits when cows were fed 173 g of CP/kg of dry matter (compared with 144 and 114 g of CP/kg of dry matter) during the first half of lactation, but they did not observe detrimental effects on production when CP was decreased to 144 g of CP/kg of dry matter during the second half of lactation. The low MUN

observed in PREC cows from cows with more than 81 DIM suggests that these cows had a limited dietary CP supply, and this fact might limit long-term milking performance as observed in Reynolds *et al*. (2016). Our study covered a relatively short period of time because the main objectives were to evaluate the precision feeding system, but Reynolds *et al*. (2016) conducted a study to assess long-term effects of dietary protein concentration (140, 160, 180 g of CP/kg of dry matter) over 3 consecutive lactations. Their results indicate that low protein diets (i.e., 140 g of CP/kg of dry matter) are more efficient, but they have detrimental effects on milk yield. This suggests a need to evaluate this precision feeding system in long-term conditions to properly assess performance data.

Estimated energy needs were covered with both diets except for early DIM cows in PREC, who needed some feed supplements to fully satisfy them. However, in general, cows overconsumed energy, even in PREC cows, in part, because when supplementing for CP using soybean pellets, cows were also supplemented for energy. However, CONV cows were fed a constant of 6.82 or 6.65 MJ/kg in period 1 and 2, respectively, in contrast to PREC cows that consumed different amounts of supplements in the milking parlour, resulting in an energy density range of the total DM consumption between 6.82 to 6.40 NE_L MJ/kg. Maltz *et al*. (2013) proposed to feed energy by adjusting caloric density of the diet between 1.59 to 1.68 NE_L weekly for 3 to 19 wk postpartum, and they succeeded in improving dairy cow performance.

The equations used to estimate the protein and energy requirements of PREC cows, decreased protein and NE concentration in the diet, and increased aNDF dietary concentrations in PREC cows. This allowed to reduce urine N excretion, but also to improve aNDF digestibility. Although the AIA recovery rate was not calculated in the present study, the ratio AIA in faeces: PMR was similar in both diets (2.44 vs 2.46 ± 0.086 in CONV and PREC fed cows, respectively). Lee *et al.* (2013) calculated recovery rate for high-producing dairy cows fed two different CP diets, and they obtained similar AIA recovery rate for both diets (86.9%). However, the ratio AIA faeces: PMR was greater in low than in high CP diet, and it resulted in greater apparent nutrient digestibilities in low than in high CP diets. In our study, we could envisage a similar impact of AIA recovery rate in both feeding systems. In the present study, nutrient digestibility was lower compared with the literature (Matins *et al*., 2022; Hynes *et al*., 2016), which was probably due to an overestimation of fecal output when using AIA as an internal marker (Morris *et al*., 2018). Rius *et al*. (2010) observed a maximal ENU when a high energy diet was combined with a low protein diet. In contrast, herein, PREC fed cows did not improve ENU. The lack of improvement might be due to the use milk total protein instead of milk true protein in N balance calculations as done in Rius *et al*. (2010) study. Although milk non-protein nitrogen (NPN) was not measured in our study, we analysed MUN, and it was greater in CONV than in PREC cows. Assuming that MUN is the main contributor to milk NPN (dePeters and Ferguson, 1992) and both measures are

correlated (Ruska and Jonkus, 2014), we could envisage more NPN concentration in CONV than in PREC cows. Both, NPN composition and milk NPN yield was increased in late lactation cows fed 165 vs 120 g of CP/kg of DM diets (Cantalapiedra-Hijar *et al*., 2014). When using the equation proposed by dePeters and Ferguson (1992) based on blood urea N and milk yield to predict NPN yield, ENU herein resulted in 19.5 and 19.1% in CONV and PREC fed cows, respectively. These values were lower than the ones reported in Table 5, but they followed the same trend. When N excretion in faeces, urine, and milk were subtracted to N intake, the negative N balance was numerically worse in CONV than in PREC cows, which was in line with the numerical BW loss observed in both feeding treatments. The difference in urinary N output between both diets was expected since almost all N ingested in excess of requirements is excreted in urine (Castillo *et al*., 2000; Cantalapiedra-Hijar *et al*., 2014). Olmos Colmenero and Broderick (2006) also reported similar percentage of urinary N excreted as dietary CP increased. The reduction in N excreted in urine represents a potential reduction of footprint and NH_3 emissions and a potential reduction in feed costs (Hynes *et al*., 2016). Although manure storage emissions were evaluated in period 2 taking manure directly from the floor of the pen, and N balance was performed in period 1, the lower total N concentration in the boxes of manure of cows fed PREC than in those on CONV, also indicates a reduction of N in the manure from PREC cows, which resulted in a numerical reduction of NH_3 gas and tendency to increase N_2O emissions. The N in urine is rapidly hydrolysed to NH_3 and then transformed to N_2O via nitrification-denitrification process (Dijkstra *et al*., 2011). As reported by Mohankumar Sajeev *et al*. (2018), the reduction of dietary CP could represent an NH_3 emission decrease by 42 ± 21%. Besides, the reduction of CP in manure also affects CH_4 emissions (Mohankumar Sajeev *et al*., 2018; Montalvo *et al*., 2013). These authors described an increase in CH_4 emissions of 20 ± 30% by each percentage-point reduction in dietary CP. In our study, PREC cows consumed a diet with a 2% points lower CP than CONV, and the manure CH_4 emissions increased 27.5% increase per every percentual-point difference in dietary CP in PREC. This could be explained by an increase in the concentration of carbohydrates of diets with low protein concentration and a decrease in fibre digestion, which tend to increase manure CH_4 production (Nampoothiri *et al*., 2015). Furthermore, the dimensions of the box used for manure storage and its depth could also influence gas emissions. The minimum depth recommended for lagoons is 2.5 m, being 2.5 – 6 m typical values (Pfost *et al*., 2000). As the depth increases, the surface can be reduced, so less emissions can be lost. Leytem *et al*. (2017) described CH_4 emissions from different studies ranging from 125 mg/m^{2}h to 8458 mg/m^{2}h, but depth was not a factor affecting the emissions. Temperature and wind were the main ones. McGinn *et al*. (2008) described NH_3 emissions in dairy cow manure stored in lagoons over summer from 3600 to 8600 mg/m^{2}h depending on the moment of the day, while Leytem *et al*. (2018) reported an annual NH_3 emission media in lagoons from 50 to 179 mg/m^{2}h with variations depending on the season (temperature, rain and wind involved), the fraction stored (liquid or

solid) and the formation of a natural crust (Grant and Boehm, 2020). Even though sampling temperature did not vary much, between 27-32ºC, temperature profile 24h was not monitored. The effect of temperature is a well-known emission factor. Season variations cause an increase in GHG during the warmer months. From 25 studies analyzed by Kupper *et al*. (2020), there was a difference of 94% between the cold-warm months.

Nutrient manure characterization of CONV and PREC fed cows resulted in a similar C, aNDF, and ADF content for both treatments, and only the ratio C to N slightly favoured PREC manure to produce more CH_4 (Dobre *et al*., 2014). Furthermore, in period 1, PREC cows showed an improvement of fibre digestibility, which might suggest a more cellulolytic microbiome that may also enhance biogas production (Sinha *et al*., 2021). CH_4 and CO_2 emission peak for CONV manure was found by day 6, and suddenly a reduction of emissions occurred. This fact can be explained by the formation of a superficial solid air-dried crust which may affect gas transportation, as it decreases superficial water content and thus, the decrease of liquid-air (atmosphere) exchanges (Aguerre *et al*., 2012; Misselbrook *et al*., 2005; VanderZaag *et al*., 2008). Although there were differences in CH_4 emission between PREC and CONV manure, both values were within the range of emission (3-25 g CH_4/m^2h) proposed by di Petra *et al*. (2019) during the first 2 wk of storage. In our study, we did not measure enteric emissions, but a prediction of the CH_4 emission factor (Y_m) was performed in cows in period 1, in which diet digestibility was assessed, using the equation described by Jaurena *et al*. (2015). No differences between feeding treatment were observed in predicted CH_4 emissions (3.19 and 3.49 ± 0.155 %GE in CONV and PREC cows, respectively). Therefore, we can only infer herein that manure from PREC cows increases CH_4 emissions during storage. Thus, if manure is used as a substrate for biogas production or if CH_4 is recovered from covered ponds, precision feeding can be considered as a good alternative, not only to reduce NH_3 emissions from manure storages and land application, but also to produce more biogas during the anaerobic fermentation, thus reducing GHG emissions to the atmosphere.

Conclusions

In conclusion, adjusting total dietary N supply based on individual animal requirements can be an effective strategy to reduce N excretion. Further research is envisaged to evaluate long-term effect of this proposed precision feeding. In addition, a potential reduction of NH_3 emissions and an increase of GHG emissions (CH_4 and CO_2) from manure of cows fed using a precision feeding strategy could be expected. This increase of CH_4 production during the storage of manure from cows fed using a precision strategy, opens a new possibility towards the integration of animal feeding systems and manure valorisation technologies to increase the sustainability of the dairy sector.

References

Aguerre, M.J., Wattiaux, M.A., Powell, J.M. 2012. Emissions of ammonia, nitrous oxide, methane, and carbon dioxide during storage of dairy cow manure as affected by dietary forage-to-concentrate ratio and crust formation, Journal of Dairy Science, Volume 95, Issue 12, 2012, Pages 7409-7416, ISSN 0022-0302, https://doi.org/10.3168/jds.2012-5340.

AOAC (Association of Official Analytical Chemists). 1990. Official methods of analysis. 15th ed. AOAC, Arlington, VA.

APHA-AWWA-WEF. 2005. Standard methods for examination of water and wastewater. 21st ed. American Public Health Association (APHA), American Water Works Association (AWWA), Water Environment Federation (WEF) Washington, DC.

Bach, A., Cabrera, V. 2017. Robotic milking: Feeding strategies and economic returns. J. Dairy Sci. 100, 1-9. https://doi.org/10.3168/jds.2016-11694.

Barros, T., Quaassdorff, M. A., Aguerre, M. J., Olmos Colmenero, J. J., Bertics, S. J., Crump, P. M., Wattiaux, M. A. 2017. Effects of dietary crude protein concentration on late-lactation dairy cow performance and indicators of nitrogen utilization. J. Dairy Sci. 100, 5434-5448. https://doi.org/10.3168/jds.2016-11917.

Bernard, J. K. 1997. Milk production and composition responses to the source of protein supplements in diets containing wheat middlings. J. Dairy Sci. 80, 938-942. https://doi.org/10.3168/jds.S0022-0302(97)76017-X.

Cantalapiedra-Hijar, G., Peyraud, J. L., Lemosquet, S., Molina-Alcaide, E., Boudra, H., Nozière, P., Orttigues-Marty, I. 2014. Dietary carbohydrate composition modifies the milk B efficiency in latte lactation cows fed low crude protein diets. Animal 8, 275-285. https://doi.org/10.1017/S1751731113002012.

Castillo, A. R., Kebreab, E., Beever, D. E., France, J. 2000. A review of efficiency of nitrogen utilization in lactating dairy cows and its relationship with environmental pollution. J. Anim. Feed Sci. 9, 1-32. https://doi.org/10.22358/jafs/68025/2000.

Comission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed.

Dalby, F.R., Guldberg, L.B., Feilberg, A., Kofoed, M.V.W. 2022. Reducing greenhouse gas emissions from pig slurry by acidification with organic and inorganic acids PloS One, 17, e0267693. https://doi.org/10.1371/journal.pone.0267693.

dePeters, E.J., Ferguson, J.D. 1992. Nonprotein Nitrogen and protein distribution in the milk of cows. J. Dairy Sci. 75, 3192-3209. https://doi.org/10.3168/jds.S0022-0302(92)78085-0.

Dijkstra, J., Oenema, O., Bannink, A. 2011. Dietary strategies to reducing N excretion from cattle: implications for methane emissions. Curr. Opin. Environ. Sustain. 3, 414-422. https://doi.org/10.1016/j.cosustt.2011.07.008.

Dobre, P., Nicolae, F., Matei, F. 2014. Main factors affecting biogas production – a overview. Rom. Biotechnol. Lett. 19, 9283-9286.

Fisher, A., Edouard, N., Faverdin, P. 2020. Precision feed restriction improves feed and milk efficiencies and reduces emissions of less efficient lactating Holstein cows without impairing their performance. J. Dairy Sci. 103, 4408-4422. https://doi.org/10.3168/jds.2019-17654.

Grant, R. H., & Boehm, M. T. 2020. Ammonia Emissions from Differing Manure Storage Facilities at Two Midwestern Free-Stall Dairies. Atmosphere, 11, 1108. https://doi.org/10.3390/atmos11101108.

Groff, E. B., Wu, Z. 2005. Milk production and nitrogen excretion of dairy cows fed different amounts of protein and varying proportions of alfalfa and corn silage. J. Dairy Sci. 88, 3619-3632. https://doi.org/10.3168/jds.S0022-0302(05)73047-2.

Hokestra, N. J., Schulte, R. P. O., Forrestal, P. J., Hennessy, D., Krol, D. J., Lanigan, G. J., Müller, C., Shalloo, L., Wall, D. P., Richards, K. G. 2020. Scenarios to limit environmental nitrogen losses from dairy expansion. Sci. Total Environ. 707, 134606. https://doi.org/10.1016/j.scitotenv.2019.134606.

Hynes, D. N., Stergiadis, S., Gordon, A., Yan, T. 2016. Effects of concentrate crude protein content on nutrient digestibility energy utilization, and methane emissions in lactating dairy cows fed fresh-cut perennial grass. J. Dairy Sci. 99, 8858-8866. https://doi.org/10.3168/jds.2016-11509.

Jaurena, G., Cantet, J. M., Arroquy, J.I., Paalladino, R.A., Wawrzkiewicz, Colombatto, D. 2015. Prediction of the Y_m fctor for livestock from on-farm accessible data. Liv. Sci. 177:52-62. https://dx.doi.org/10.1016/j.livsci.2015.04.009.

Kupper, T., Häni, C., Neftel, A., Kincaid, C., Bühler, M., Amon, B., VanderZaag, A. 2020. Ammonia and greenhouse gas emissions from slurry storage - A review. Agriculture, Ecosystems & Environment, Volume 300, 2020, 106963, ISSN 0167-8809, https://doi.org/10.1016/j.agee.2020.106963.

Law, R.A., Young, F.J., Patterrson, D.C., Kilpatrick, D.J., Wylie, A.R.G., Mayne, C.S. 2009. Effect of dietary protein content on animal production and blood metabolites of dairy cows during lactation. J. Dairy Sci. 92, 1001-1012. https://doi.org/10.3168/jds.2008-1155.

Lee, C., Hristov, A.N. 2013. Short communication: Evaluation of acid-insoluble ash and indigestible neutral detergent fiber as total-tract digestibility markers in dairy cows fed corn silage-based diets. J. Dairy Sci. 96, P5295-5299. https://doi.org/10.3168/jds.2012-6442.

Levy, P., Cowan, N. J., Van Oijen, M., Famulari, D., Drewer, J., Skiba, U. 2017. Estimation of cumulative fluxes of nitrous oxide: uncertainty in temporal upscaling and emission factors: estimation of cumulative fluxes of nitrous oxide. Eur. J. Soil Sci. 68. https://doi.org/10.1111/ejss.12432.

Leytem, A.B., Bjorneberg, D.L., Koehn, A.C., Moraes, L.E., Kebreab, E., Dungan,R.S. 2017. Methane emissions from dairy lagoons in the western United States, Journal of Dairy Science, Volume 100, Issue 8, 2017, Pages 6785-6803, ISSN 0022-0302, https://doi.org/10.3168/jds.2017-12777.

Leytem, A. B., Bjorneberg, D. L., Rotz, C. A., Moraes, L. E., Kebreab, E., Dungan, R. S. 2018. Ammonia emissions from dairy lagoons in the western U.S., Trans. ASABE, 61, 1001–1015, https://doi.org/10.13031/trans.12646.

Linn, J. 1988. Factors Affecting the Composition of Milk from Dairy Cows in Designing Foods: Animal Product Options in the Marketplace. National Academies Press, Washington, US). https://www.ncbi.nlm.nih.gov/books/NBK218193/

Kupper, T., Häni, C., Neftel, A., Kincaid, C., Bühler, M., Amon, B., VanderZaa, A. 2020. Ammonia and greenhouse gas emissions from slurry storage – A revie., Agric. Ecosyst. Environ. 300, 106963. https://doi.org/10.1016/j.agee.2020.106963.

Maltz, E., Barbosa, L. F., Bueno, P., Scagion, L., Kaniyamattam, K., Greco, L. F., De Vries, A., Santos, J. E. P. 2013. Effect of feeding according to energy balance on performance, nutrient excretion, and feeding behavior of early lactation dairy cows. J. Dairy Sci. 96, 5249-5266. https://doi.org/10.3168/jds.2013-6549.

Martins, L. F., Oh, J., Harper, M., Melgar, A., Räisänen, S. E., Chen, X., Nedelkov, K., Karnezos, T. P., Hristov, A. N. 2022. Effects of an exogenous enzyme preparation extracted from a mixed cultue of *Aspergillus* spp. On lactational performance, metabolism, and 67eteorologic67 in primiparous and multiparous cows. J. Dairy Sci. 105, 7344-7353. https://doi.org/10.3168/jds.2022-21990.

McGinn, S.M., Coates, T., Flesch, T.K., Crenna. B. 2011. Ammonia emission from dairy cow manure stored in a lagoon over summer. Can. J. Soil Sci. 88, 611-615. https://doi.org/10.4141/CJSS08002.

Misselbrook, T.H., Powell, J.M., Broderick, G.A., Grabber, J.H. 2005. Dietary manipulation in dairy cattle: Laboratory experiments to assess the influence on ammonia emissions J. Dairy Sci. 88,1765-1777.

Mohankumar Sajeev, E.P., Winiwarter, W., Amon, B. 2018. Greenhouse Gas and Ammonia Emissions from Different Stages of Liquid Manure Management Chains: Abatement Options and Emission Interactions. J. Environ. Qual. 47, 30-41. https://doi.org/10.2134/jeq2017.05.0199. PMID: 29415114.

Montalvo, G., Morales, J., Pineiro, C., Godbout, S., Bigeriego, M. 2013. Effect of different dietary strategies on gas emissions and growth performance in post-weaned piglets. Span. J. Agric. Res. 11, 1016–1027. Doi:10.5424/ sjar/2013114-3185.

Moore, S. M., King, M. T. M., Carpenter, A. J., DeVries, T. J. 2020. Behavior, health, and productivity of early-lactation dairy cows supplemented with molasses in automated milking systems. J. Dairy Sci. 103, 10506-10518. https://doi.org/10.3168/jds.2020-18649.

Morris, D. L., Rebelo, L. R., Dieter, P. A., Lee, C. 2018. Validating intrinsic markers and optimizing spot sampling frequency to estimate fecal outputs. J. Dairy Sci. 101, 1-10. https://doi.org/10.3168/jds.2018-14717.

Nampoothiri, V.N., Mohini, M., Thakur, S. S., Mondal, G. 2015. Influence of diet on methane and nitrous oxide emissions from cattle manure. Asian J. Atmos. Environ. 9, 187–193. https://doi.org/10.5572/ajae.2015.9.3.187.

Olmos Colmenero J. J., Broderick, G. A. 2006. Effect of dietary crude protein concentration on milk production and nitrogen utilization in lactating dairy cow. J. Dairy Sci. 89, 1704-1712. https://doi.org/10.3168/jds.S0022-0302(06)72238-X.

di Perta, E. S., Cervelli, E., Faugno, S., Pindozzi, S. 2019. Monitoring of NH3 and CH4 emissions from dairy cows under storage conditions. IEEE International Workshop on Metrology for Agriculture and Forestry. Pages 35-39. https://doi.org/10.1109/MetroAgriFor.2019.8909270.

Pfost, D. L., C. D. Fulhage, Rastorfer D. 2000. Anaerobic lagoons for storage/treatment of livestock manure. University of Missouri Extension Publ. No. EQ 387. University of Missouri–Columbia.

Postigo C., Ginebreda, A., Barbieri, M.V., Barceló, D., Martín-Alonso, J., de la Cal, A., Boleda, M. R., Otero, N., Carrey, R., Solà, V. Queralt, E. Isla, E. Casanovas, A. Frances, G., López de Alda, M. 2021. Investigative monitoring of pesticide and nitrogen pollution sources in a complex multi-stressed catchment: The lower Llobregat River basin case study (Barcelona, Spain), Sci. Total Environ. 755, 142377.

Reynolds, C. K., Crompton, L. A., Humphries, D. J., Jones, A. K. 2016. Long term implications of feeding low protein diets to first lactation dairy cows. *In:* J. Skomial, and H. Lapierre (eds.) Proceedings of the 5th International Symposium on Energy and Protein Metabolism, EAAP Publ. No. 137. Wageningen Academic Publishers, The Netherlands. Pages 263-264.

Rius, A. G., McGuilliard, M. L., Umberger, C. A., Hanigan, M. D. 2010. Interactions of energy and predicted metabolizable protein in determining nitrogen efficiency in the lactating dairy cows. J. Dairy Sci. 93, 2034-2043. https://doi.org/10.3168/jds.2008-1777

Ruska, D., Jonkud, D. 2014. Crude protein and non-protein nitrogen content in dairy cow milk. Journal of Latvian University of Life Sciences and Technologies. 32, 327. https://doi.org/10.2478/plua-2014-0011.

Sales, J., Janssens, G. P. J. 2003. Acid-insoluble ash as a marker in digestibility studies: a review. J. Anim. Feed Sci. 12, 383-401. https://doi.org/10.22358/jafs/67718/2003.

Sinha, D., Banerjee, S., Mandal, S., Basu, A., Banerjee, A., Balachandran, S. Mandal, N. C., Chaudhury, S. 2021. Enhanced biogas production from Lantana camara via bioaugmentation of cellulolytic bacteria. Bioresour. Technol. 340, 125652. https://doi.org/10.1016/j.biortech.2021.125652.

Talke, H., Schubert, G. E. 1965. Enzymatic urea determination in the blood and serum in the Warburg optical test. Wiener klinische Wochenschrift. 43, 174-175. https://doi.org/10.1007/BF01484513.

Torrellas, M., Burgos, L., Tey, L., Noguerol, J., Riau, V., Palatsi, J., Antón, A., Flotats, X. Bonmatí, A. 2018. Different approaches to assess the environmental performance of a cow manure biogas plant. Atmos. Environ. 177, 203-213. https://doi.org/10.10167j.atmosenv.2018.01.023.

VanderZaag, A.C., Gordon, R.J., Glass, V.M., Jamieson R.C. 2008. Floating covers to reduce gas emissions from liquid manure storages: A review Appl. Eng. Agric., 24, 657-671.

VanderZaag, A., Amon, B., Bittman, S., Kuczynski, T. 2015. Ammonia abatement with manure storage and processing techniques. In S. Reis, C. Howard, and M. A. Sutton in Costs of ammonia abatement and the climate co-benefits. Pages 75-112. Springer Netherlands.

Van Empel, M., Makkar, H. P. S., Dijkstra, J., Lund, P. 2016. Nutritional, technological and managerial parameters for precision feeding to enhance feed nutrient utilization and productivity in different dairy cattle production systems. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources, 11. https://doi.org/10.1079/PAVSNNR201611037

van Rossum, G., Drake, Jr. F. L. 1995. Python reference manual. Centrum voor Wiskunde en Informatica Amsterdam, The Netherlands.

Van Soest, P. J., Robertson, J. B., Lewis, B. A. 1991. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583-3597.

Valadares, R. F. D., Broderick, G. A., Valadares Filho, S. C. Clayton, M. K. 1999. Effect of replacing alfalfa silage with high moisture corn on ruminal protein synthesis estimated from excretion of total purine derivatives. J. Dairy Sci. 82, 2686-2696.

Zhang Q., Ren, F., Li, F., Chen, G., Yang, G., Wang, J., Du, K., Liu, S., Li, Z. 2020. Ammonia nitrogen sources and pollution along soil profiles in an in-situ leaching rare earth ore. Environ. Pollut. 267, 115449. https://doi.org/10.1016/j.envpol.2020.115449.

Chapter 6 – Identification of bacteriophages linked to the activity of ruminal hyper-ammonia producing bacteria (HAP)

CRediT authorship contribution statement

L. Morey: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft, Writing-Review & Editing. **E. Garcia:** Conceptualization, Methodology, Writing-Review & Editing. **V. Riau:** Writing-Review & Editing, Support on Formal Analysis, Supervision, Project administration, Funding Acquisition. **M. Terré:** Writing – Writing-Review & Editing, Supervision, Funding Acquisition. **S. Escribano**: Support on Formal Analysis. **M. Llagostera:** Methodology, Validation, Supervision. **M.A. Cortés:** Conceptualization, Methodology, Validation, Investigation, Software, Supervision.

Abstract

Nutrient efficiency for ruminants depends, among other factors, on the type of feed included in the diet, that can affect rumen microbiome. Nitrogenous compounds are degraded first to NH_3 to be part of the microbial produced proteins. Nevertheless, when the feed protein exceeds the ruminal microbiota requirements, especially when there is protein overfed in intensive production systems, there is also an excess of NH_3 production.

Although HAP such as *Clostridium aminophilum, Clostridim sticklandii*, and *Peptostreptococcus anaerobius*, represent only 5% in cow's ruminal microbiota, they significantly contribute to NH_3 production in the rumen. The control of HAP population in the rumen is envisaged as a potential strategy to improve the efficiency of N. Several studies using plants and plant extracts, such as hops or tannins or via diet modifications, have been proposed to reduce their activity. Alternatively, bacteriophages, viruses capable of targeting and infecting one specific bacterial host, are present in the ruminal fluid targeting most of the rumen bacteria species. Thus, the use of specific bacteriophages against HAP could be used to reduce the HAP population in the rumen.

Ruminal fluid from dairy cows at 4 different physiological stages (early lactation, late lactation, dry-off, and growing heifers) were collected and cultivated with a specific HAP media to select and isolate different colony morphologies. The isolated colonies were tested to determine their NH_3 production and identified by 16S rDNA sequencing. *Clostridium sporogens*, *Terrisporobacter glycolicus* (*Clostridium glycolicum*), *Megasphaera elsdenii, Clostridium argentinense, Stretococcus sp., Prevotella ruminicola, and Acidaminococcus fermentans* were identified as HAP. Bacteriophages from the same ruminal fluid were concentrated by centrifugation and ultracentrifugation and were inoculated with each isolated HAP colony through double layer methodology to test their affinity. However, it was not possible to detect a reduction in HAP growth, showing that the use of bacteriophages to reduce HAP population of rumen needs to be further improved or approached differently.

Introduction

The emission of nitrogenous compounds (NH_3 and N_2O) in the dairy and meat bovine industry represents a problem in terms of environmental sustainability and pollution. Over 70% of GHG and NH_3 emissions from ruminant livestock is related to milk, beef, and mutton (Behera *et al.*, 2013; Ripple *et al.*, 2014), and with the continuous increase of cow derived products demand, the environmental contamination problems are becoming a significant issue (Tan *et al*., 2021).

NH_3 is one of the major gases emitted by livestock manure, 80-90% of total NH_3 emissions (Uwizeye *et al.*, 2020), and has a significant effect on human health by combining with organic volatile compounds to form aerosols (Ti *et al.*, 2019). This emitted NH_3 can affect air quality, cause water and soil acidification when it is deposited on land or water bodies, eutrophication, and loss of biodiversity (Zhang *et al.*, 2021). Also, NH_3 plays an important role in GHG emissions, as it can be transformed into N_2O, one of the most influential GHG, by ammonia-oxidizing bacteria (Prosser *et al*., 2020).

High quantities of N_2O are related with the N use efficiency in the animal, manure, soil, and crop fertilization chain (Singh *et al.*, 2010), and NH_3 emissions from manure are mainly coming from urea excretion, which is highly volatile (Tan *et al*., 2021). Specifically in ruminants, the nitrogen use efficiency is lower than the monogastric animals, 25% compared to 46% for swine (Casamiglia *et al*., 2010; Millet *et al*., 2018) leading to waste nitrogen and favoring environment pollution.

In addition, nutrient efficiency for ruminants also depends on a wide variety of other factors including physiological cow status, digestive function, metabolic partitioning, genetics, and the type of feed in diet (Bach *et al*., 2020), that modifies rumen microbiome (Hartinger *et al*., 2018). True protein is decomposed to peptides, oligopeptides, then into smaller peptides and finally into amino acids. One small part of the amino acids is directly used for protein synthesis, but the major part is deaminated first to NH_3 to be part of the microbial produced proteins (Firkins *et al*., 2007; Shen *et al.*, 2018). However, when the feed protein exceeds the ruminal microbiota necessities, especially when there is protein overfed in intensive production, there is an excess of NH_3 that will be excreted in the form of urea (Bach *et al*., 2005; Tan *et al*., 2021).

The entire deamination process involves a huge variety of microorganisms including mainly proteolytic and peptidolytic bacteria, ureolytic bacteria and deaminating bacteria (Hartinger *et al*., 2018), and only the last group degrades amino acids. Bladen *et al*., (1961) indicated that low NH_3 production bacteria were the main ruminal NH_3 producers. Nevertheless, years later, Chen and Russell, (1989) proclaimed another type of ruminal bacteria, which were less abundant but with a high NH_3 production capacity, commonly known as HAP. This finding was later confirmed by many other authors (Eschenlauer *et al*., 2002.; Flythe and Andries, 2009; Harlow *et al*., 2016).

Therefore, several studies have been done to reduce ruminal NH_3 production and reducing HAP activity. Tan *et al*., (2021) presented nutritional strategies to improve nitrogen retention and reduce its emissions using plants or their extracts in diets such as essential oils or tannins. The addition of essential oils in diet, such as clove oil and origanum oil, reduced the presence of proteolytic bacteria, decreasing the level of NH_3 on excreta. Tannins increase the pH of the rumen and in consequence enhance the abundance of *Ruminococcus* and *Fibrobacter*, improving nutrient digestibility and microbial protein synthesis. The reduction of CP in ruminant diets, as it has been demonstrated in Chapter 5, or starch proportion in feed can reduce N emissions from manure. Moreover, Flynthe *et al*., (2017) and Harlow *et al*., (2016), studied the use of hops (*Humulus lupulus* L.) and spent yeast from craft breweries (with hops) in cow's diet concluding that the antimicrobial effect due to the α and β acids reduced the HAP activity and NH_3 production.

Another approach to reduce the HAP and the associated NH_3 production could be the use of bacteriophages or phages. Bacteriophages are viruses that infect bacteria, which are highly specific. Lytic phages are capable to kill the infected bacteria via intracellular lysis (Luo *et al*., 2018). The two main advantages of using phages as antibacterial agents are: i) low side effects and disruption of microflora due to the phage specificity; ii) lack of bacterial resistance, associated with antibiotics (Moodley *et al*., 2019). Nowadays, the use of phage therapy is only approved in Russia, Georgia, and Poland, and for some specific cases in the US. In Europe it is not yet allowed due to they are not yet satisfying the regulatory standards (Naureen *et al*., 2020) but they are foreseen to be used in the near future, as the FDA (Food and Drug Administration) is open to their regulation and the National Institutes of Health (NIH) recently awarded studies of phage therapy (Barron, 2022).

Phages coexist with bacteria and consequently a large and diverse number of bacteriophages have been found in the ruminal fluid (Gilbert *et al*., 2017; Klieve *et al*., 2004). These phages have been studied and identified using different techniques such as electron microscopy, pulsed field gel electrophoresis, culture-based techniques, and metagenomics. Gilbert *et al*., (2017), Gilbert *et al*., (2020), and Klieve *et al*., (1988) described phages infecting the following bacteria genera: *Ruminococcus, Streptococcus, Bifidobacteriu, Magnoovum, Methanobrevibacter, Prevotella, Butyrivibrio, Lactobacillus, Eubacteriu, Selenomonas, Quinella, Bacteroides, Serratia,* and *Bifidobacterium*.

The hypothesis of this chapter is that the use of concentrated bacteriophages from the ruminal fluid could reduce ruminal NH_3 production by infecting and reducing HAP population isolated from the same ruminal fluid.

Materials and methods

Ruminal liquid collection

Two batches of ruminal fluid samples from four different dairy cow physiological stages: early lactation (M1), late lactation (M2), dry-off (M3), and growing heifers (M4), were collected in the experimental dairy cow station (EVAM) in Monells, Spain, using a rumen fluid scoop, as described in Geishauser *et al*., (2012) in two different periods. Ruminal fluid was collected from 4 cows fed under a unique TMR for each physiological stage and pooled together to have 100-200 mL of ruminal fluid to isolate both HAP and bacteriophages presents there. The ruminal fluid was stored in 50 mL centrifuge tubes to be transported in a thermal box, minimizing change of temperature.

Rumen cultivation

200 µL of each collected ruminal fluid were cultivated, in 20 mL sealed flasks, with 10 mL liquid culture medium based on Chen & Russell, (1990) containing 15 g/L of Trypticase or Casamino Acids, as nitrogen source, 64 mg/L of $CaCl_2 \cdot H_2O$, 100 mL/L of salt solution, 2.9 mL of volatile acids mix solution, 600 mg/L L-Cysteine hydrochloride, 1 mL/L resazurin, 0.1 ml/L hemin, 2.5 mL glucose concentrated as it is indicated in Table 6. The pH of the medium was adjusted between 6-7 with 10N NaOH and the medium was sterilized at 121 ºC during 20 min at the autoclave. After the autoclave, the vitamins were added, previously filtrated through a 0.45 µm pore filter, to avoid their denaturalization.

Table 6. Solutions and vitamins concentration.

Salt solution	g/L
K_2HPO_4	2.92
KH_2PO_4	2.4
Na_2SO_4	4.8
NaCl	4.8
$MgSO_4 \cdot 7H_2O$	1
Volatile Acids Mix	mL
Acetic	16
Propionic	6
Butyric	3.1
Valeric	1
Isovaletic	1
Isobutyric	1
2-Metyl-butytic	1
Vitamines	mg/L
Pyridoxamine dihydrochloride	20
Riboflavin	20
Thiamine hydrochloride	20
Nicotinamide	20
Calcium pantothenate	20
Lipoic acid	10
Para aminobenzoic acid	5
Folic acid	5
Biotin	0.25
Coenzyme B12	5
Hemin	10mg/ml
Glucose	20%
Resazurin	0.1%

For solid media, 17 g/L of agar were added before autoclaving. To dissolve hemin 10N NaOH was added, and for lipoic acid and para-aminobenzoic acid, ethanol 100%.

The liquid culture was incubated in anaerobic conditions, using the Gas-pak system® (Becton, Dickinson and company; New Jersey, USA), during 3-5 days at 38 ºC, until turbidity was evident. Ruminal fluid from each cow stage was cultivated separately and a control media, without ruminal fluid, was added to check for contamination.

The liquid cultures were then inoculated in solid media plates to visually identify and isolate different colonies through the streaking technique. The isolated colonies, first separated by morphology and then by 16S rDNA sequencing, were preserved in cryopearls with anaerobe formulation Protect Select (Technical Service Consultants Ltd., UK) at -80ºC.

Anaerobiosis conditions and media modifications

In order to identify a higher variety of species, the anaerobic cultivation conditions were improved with the second batch of samples. At this time, Argon gas was introduced to the ruminal fluid cultivated in liquid media to remove all presence of oxygen inside the flasks. Moreover, as almost all the isolated bacteria from the first experiment were grown with trypticase, and only one with casamino acid, it was decided to only utilize trypticase as N source in the second assay.

Selection of the HAPs

The isolated colonies were cultivated in the same liquid media to analyze their NH_3 production. Pure colonies were inoculated to 20 mL flasks with 10 mL of the liquid culture media, together with a control, without any colony, and incubated 5 days at 38 ºC under anaerobic conditions, following the previously described methodology.

Initial and final media samples were taken to analyze, after centrifugation to determine only the soluble NH_3, total ammonia nitrogen with a distillation unit Buchi® ((BUCHI Labortechnik GmbH, The Netherlands) through the total ammonia nitrogen method (method 4500-NH3-C; APHA, 2005). The concentration of $N\text{-}NH_4^+$ was transformed to nmol NH_3/min/mg protein to compare with literature. Colonies with $N\text{-}NH_4^+$ production lower than the control were rejected.

Sequencing 16S rDNA

The DNA extraction of the isolated colonies was performed with DNA genomic kit (Dneasy Blood & Tissue Kit, QIAEGEN N.V., The Netherlands), amplified by PCR with a Thermal Cycler (SimpliAmpTM Thermal Cycler, Thermo Fisher Scientific Inc., USA), and purified with a clean-up kit (QIAquick PCR Purification Kit, QIAGEN N.V., The Netherlands).

The sequencing of all DNA fragments was determined by Macrogen Inc., on an ABI 3730XL sequencer.

Bacteriophages concentration and visualization

To concentrate bacteriophages from the ruminal fluid of each different lactating phase and growing heifers, individual samples of each animal were mixed by type of animal, and it was centrifuged at 10,000 rpm for 10 min. The supernatant was collected, and the process was repeated 4 times. Final supernatants were filtered through 0.45 µm polyester sulfone membrane filters. The filtered supernatants were then concentrated 16 times by ultracentrifugation (Optima TM XPN-100, Beckman, California, USA) with a 90Ti rotor (Beckman, California, USA) at 68.584 g for 2h and 15 ºC.

The resulting pellets were dissolved in 0.5 mL of $MgSO_4$ 10mM, agitated at 200 rpm and room temperature overnight. The obtained bacteriophage suspensions were filtered again through 0.45 µm polyester sulfone membrane filters.

The filtered suspensions were observed by transmission electron microscopy (TEM), to determine the presence of bacteriophages in the samples, at "Servei de Microscopia" (Microscopy service) of the Universitat Autònoma de Barcelona. Before the observation, the samples were tincted with uranyl acetate 2%.

Bacteriophages-HAPs interaction

The standard Double Layer Agar (DLA) method visualization was used to determine if any of the obtained bacteriophages from the rumen could lyse the isolated HAP. The method is based on the formation of lytic plaques (degradation halos) over the bacterial cultures.

A variation of the DLA method of Kropinski *et al*. (2009) was followed to prepare double layers of Agar and bacterial cultures. 3 mL of 0.7% agar was mixed with 0.2 mL of liquid culture of each isolated HAP, on top of a 1.5% agar plaque. The bacteriophages were not mixed with the bacteria. As different stage cow rumen bacteriophages were collected, drops of 10 µL of each one were placed on top of the soft agar for each isolated HAP. Plaques were incubated in anaerobiosis at 38ºC during 5 days before their observation.

Moreover, an *in vitro* assay (Figure 20), in which bacteriophages content from 40 mL of ruminal fluid were concentrated to be inoculated with the liquid growing media for HAP, was performed incubating the vials in anaerobiosis for 4 days at 38ºC in a heated agitation plate (VWR International, PA, USA) per triplicate and using a control with no bacteriophages addition. TAN was determined at the beginning and at the end of the assay to assess bacteriophages efficiency in reducing NH_3 production.

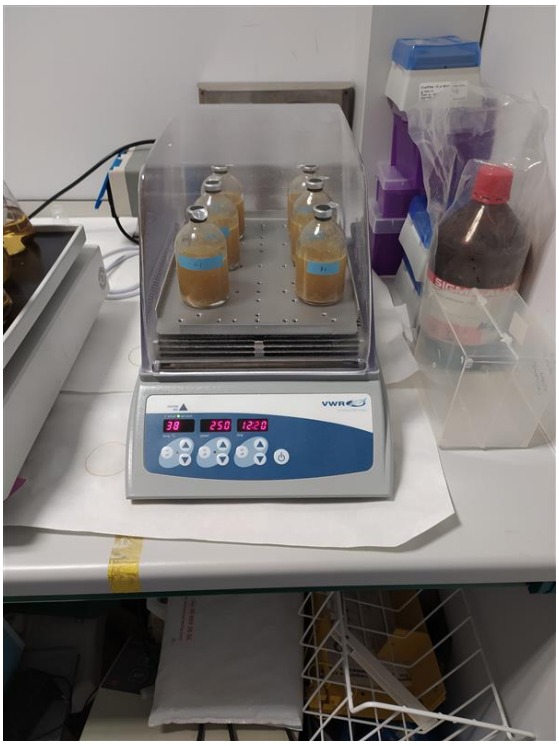


Figure 20. *In vitro* assay of HAP growing media and ruminal bacteriophages concentrate.

Results

HAP isolation and identification

Colonies from the M2, M3 and M4 ruminal fluids were isolated and identified through sequencing the 16S rDNA gene. Their NH_3 production was determined to classify them into HAP. However, no bacteria could be isolated from M1. Table 7 shows the isolated colonies with their NH_3 production.

Table 7. NH_3 production of the 16S rDNA sequenced bacteria of the isolated colonies during the first assay.

Ruminal Fluid	16 S	NH_3 Production (nmol/min/mg protein)
Late Lactation (M2)	*Clostridium botulinum /C. sporogenes*	876
	Sharpea azabuensis/Lachnospiraceae	-
	Terrisporobacter glycolicus (Clostridium glycolicum)	726
	Terrisporobacter glycolicus (Clostridium glycolicum)	626
	Terrisporobacter glycolicus (Clostridium glycolicum)	617
	Clostridium sporogenes.	576
Dry (M3)	*Streptococcus sp.*	840
	Bisgaard Taxon 10/Actinobacillus succinogenes	146
Heifers (M4)	*Megasphaera elsdenii*	682
	Prevotella ruminicola	348
	Enterococcus avium	357
	Clostridium sporogenes.	617
	Clostridium sporogenes.	583

Late lactation cows (M2) presented mostly *Clostridium* bacteria with high concentration of produced NH_3, with values ranging between 576-876 nmol NH_3/min/mg protein. *Sharpea azabuensis* was also identified but its production was the same as the control. For M3, *Streptococcus* and *Actinobacillus* presented a production of 840 nmol NH_3/min/mg protein and 146 nmol NH_3/min/mg protein, respectively. Finally, the highest variability of bacterial genera was observed in M4 samples. *Megasphaera*, *Prevotella*, *Enterococcus* and *Clostridium* were isolated with NH_3 production ranging between 357-682 nmol NH_3/min/mg protein. All the mentioned bacteria were growth with Trypticase as nitrogen source except for *Enterococcus* which was growth with Casamino acid.

HAP isolation and identification with Argon inoculation

In this occasion, bacteria from all cow stages ruminal fluid (M1, M2, M3, M4) were isolated and identified following the same steps as in the previous assay. Table 8 shows the sequenced bacteria identification and their NH_3 production.

Table 8. NH_3 production of the 16S rDNA sequenced bacteria of the isolated colonies during the second assay.

Ruminal Fluid	16 S	NH_3 Production (nMol/min/mg protein)
Early Lactation (M1)	*Clostridium argentinense/BLAST: C. subterminale/C.argentinense*	570
	Staphylococcus warneri	13
	Clostridium sporogenes	419
	Clostridium sporogenes	525
Late Lactation (M2)	*Acidaminococcus fermentans*	602
	Clostridium sporogenes	440
	Streptococcus gallinarum	-
Dry (M3)	*Clostridium sporogenes*	426
	Clostridium argentinense/schirmacherense	722
	Clostridium argentinense/BLAST: C. subterminale/C.argentinense	614
	Streptococcus gallinarum	44
	Clostridium sporogenes	472
Heifers (M4)	*Clostridium sporogenes*	424

In M1 samples bacteria from the genera *Clostridium* and *Staphylococcus* were observed. *Staphylococcus warneri* with a negligible NH_3 production, and *Clostridium argentinese* and *sporogenes* with an NH_3 production between 419 to 570 nmol NH_3/min/mg protein (Table 8). M2 bacteria genera were different from the first experiment, except for *Clostridium*. *Acidaminococcus*, *Clostridium* and *Streptococcus* were isolated during the second assay, with ammonia productions values ranging between 440 and 602 nmol NH_3/min/mg protein, except for *Streptococcus*, with a zero-production compared to the control. Dry cows' ruminal fluid (M3) showed two species of *Clostridium*: *Clostridium sporogenes* with an NH_3 production of 426 nMol NH_3/min/mg protein and *Clostridium argentinense* with an NH_3production ranging between 614-722 nmol NH_3/min/mg protein. *Streptococcus* was also isolated from M3 samples with a marginal production of NH_3. Regarding M4, only one colony of *Clostridium sporogenes* was isolated this time, with a production of 424 nmol NH_3/min/mg protein.

Bacteriophages Identification and efficiency

In Figures 21 (M1 samples), 22 (M2 samples), 23 (M3 samples), and 24 (M4 samples) images of the bacteriophages isolated from rumen observed under TEM are shown. Different morphologies of bacteriophages mostly with long tail compatible with Myoviridae or Siphoviridae with

contractile and noncontractile long tail were observed. In some of the M2 and M4 samples, morphologies compatible with short tail *Podoviridae* family were found. In terms of quantity, M3 presented the smallest number of bacteriophages.

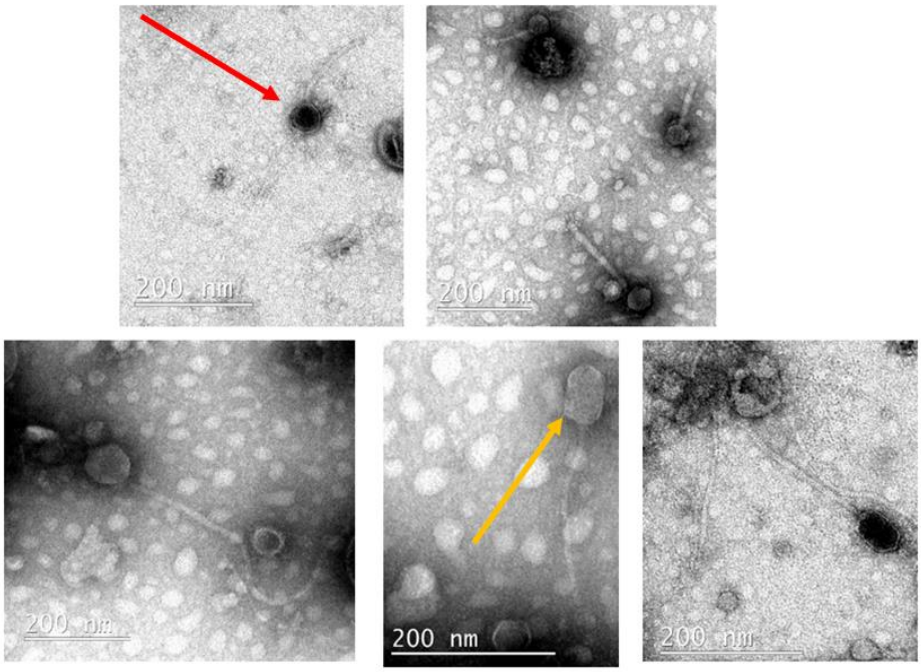


Figure 21. Transmission electron microscopy images of the ruminal fluid samples M1-Early Lactation, concentrated by ultracentrifugation. Scale bars are indicated in each image. The red arrow indicates a *Siphoviriae* bacteriophage. The yellow arrow indicates a *Myovirdae* bacteriophage.

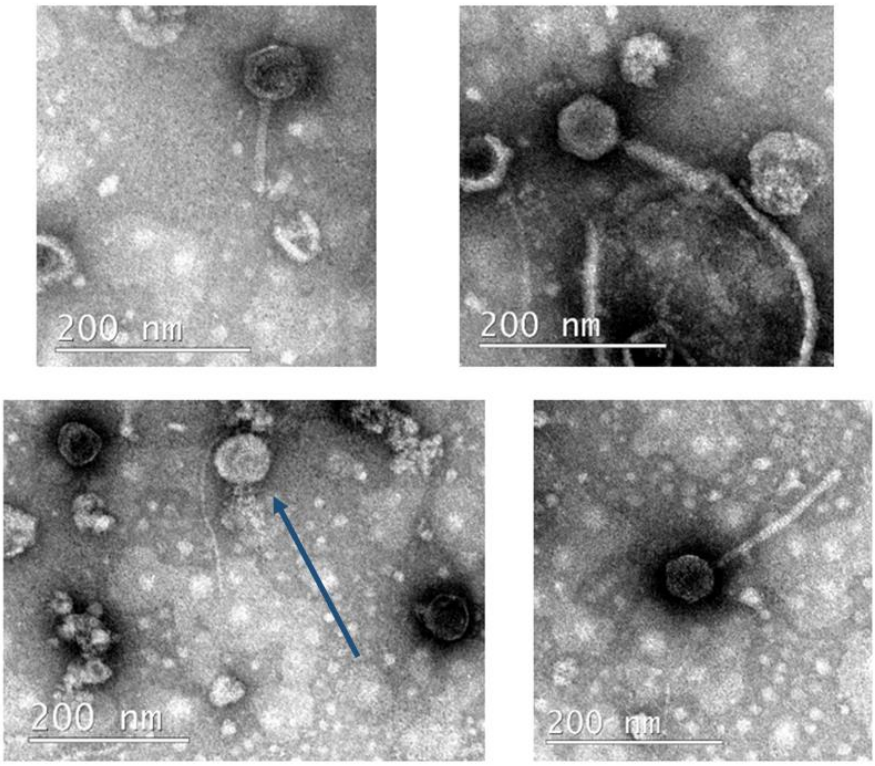


Figure 22. Transmission electron microscopy images of the ruminal fluid samples M2-Late Lactation, concentrated by ultracentrifugation. Scale bars are indicated in each image. The blue arrow pointed to a *Podoviridae* bacteriophage.

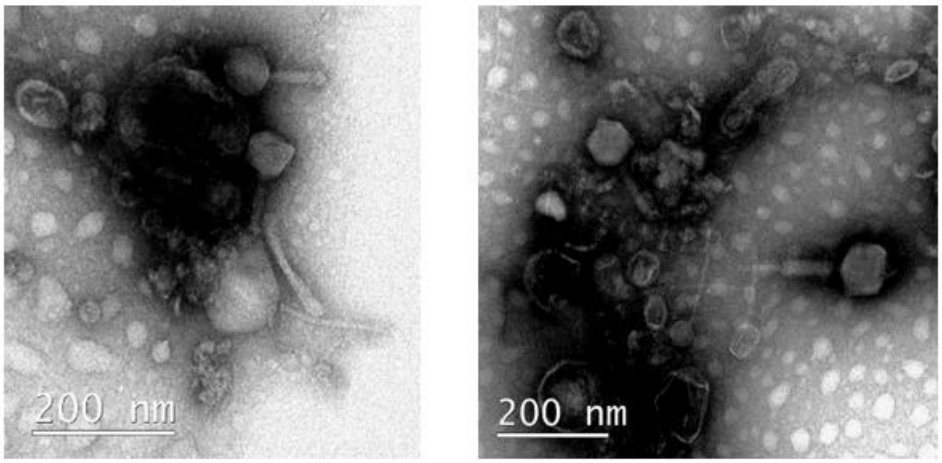


Figure 23. Transmission electron microscopy images of the ruminal fluid samples M3-Dry, concentrated by ultracentrifugation. Scale bars are indicated in each image.

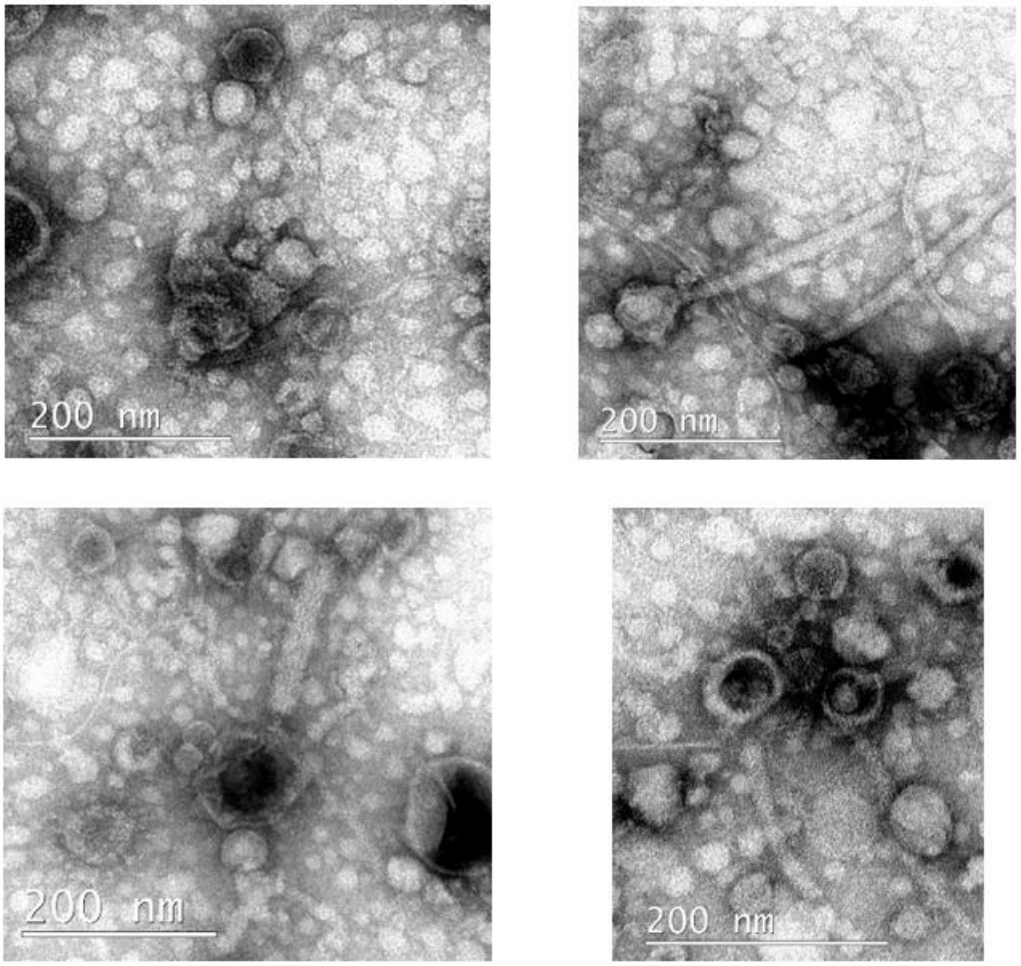


Figure 24. Transmission electron microscopy images of the ruminal fluid samples M4-Heifers, concentrated by ultracentrifugation. Scale bars are indicated in each image.

After the DLA, combining the isolated HAP with the bacteriophage's solution, no interaction or clear ring appeared on the incubated plaques.

During the *in vitro* assay of liquid media, we could not observe any significant differences between the control and the bacteriophages media (Table 9), both with $N\text{-}NH_4^+$ production increase over 600%.

Table 9. $N\text{-}NH_4^+$ production of the liquid *in vitro* assay with concentrated bacteriophages.

	Control	Bacteriophages	SEM	p-value
Initial $N\text{-}NH_4^+$ (mg/kg)	97	97	1.2	0.85
Final $N\text{-}NH_4^+$ (mg/kg)	766	745	11	0.24
TAN variation (%)	693	668	16	0.34

Discussion

HAP identification

Deamination of amino acids in the rumen is performed by 2 groups of bacteria, and both produce NH_3. The first one presents a major number of species with an activity of 10-20 nmol NH_3/min/mg protein. This includes *Butyrivibrio fibrisolvens*, *Prevotella ruminicola* and *Megasphaera elsdenii* (Hartinger *et al*., 2018). The second group represents the HAP, with an activity higher than 300 nmol NH_3/min/mg protein according to Hartinger *et al*., (2018) or 250 to Eschenlauer *et al*., (2002).

In previous works, *Clostridium aminophilum*, *Clostridium sticklandii*, *Peptostreptococcus anaerobius*, *Clostridium* sp., *Eubacterium* sp., *Aciadminococcus fermentans*, *Clostridium sticklandii*, *Clostridium bifermentans*, and *Clostridium argentinense* were isolated from ruminal fluid and identified as HAP (Atwood *et al*., 1998; Eschenlauer *et al*., 2002; Flythe *et al*., 2017; Hartinger *et al*., 2018; Paster *et al*., 1993), with NH_3 productions between 552-945 mmol NH_3/min/mg protein. In this experiment, except for *Shapea azabuensis*, *Streptococcus gallinarum*, *Actinobacillus succinogenes and Staphylococcus warneri*, all the isolated colonies produced more than 300 mmol NH_3/min/mg protein (Tables 7 & 8), the stablished NH_3 production limit to be considered HAP by Hartinger *et al*., (2018). *Staphylococcus warneri* was considered a contamination, since it is not a ruminal bacterium but a common human and animal skin bacterium (Kunuparthy *et al*., 2020), who proved to growth in a specific media for HAP. *Actinobacillus succinogenes* is a well-known succinic acid-producing bacteria (Taraka and Doddapaneni, 2021) from the bovine rumen, *Streptococcus gallinarum* is a common intestinal bacterium (Taban *et al.,* 2014) also found as mastitis pathogen (Yun *et al.*, 2020), and *Sharpea azabuensis* is a ruminal bacterium that metabolizes linoleic acid (Dewanckele *et al*., 2019). Regarding *Clostridium* species, *Clostridium sporogens, Clostridium glycolicum* and *Clostridium argentinense* were isolated and presented as HAP, as they produced between 419-876 nmol NH_3/min/mg protein.

Streptococcus sp. were found to produce a high quantity of NH_3 (840 nmol NH_3/min/mg protein). Despite literature does not describe any *Streptoccoccus* as HAP, among *Streptococcus sp.*, *S. bovis* was mentioned to be a deaminating bacteria (Hartinger *et al*., 2018). As it is difficult to differentiate *Streptoccoccus* because of its imperfect phenotypic attributes (Dekker and Lau, 2016), DNA sequencing for *Streptococcus sp.* should be improved to better assess the high-producing *Streptococci* specie found in the present study.

Megasphaera elsdenii and *Prevotella ruminicola* are described as a deaminating bacteria, but not in the HAP group. However, in this study, the production of NH_3 was higher than 300 nmol NH_3/min/mg protein, and even comparable to *Clostridium*, (682 nmol NH_3/min/mg protein) in

the case of *M. elsdenii*. Even though Hartinger *et al*., (2018) defined that some strains of *M. elsdenii* have NH_3 production rates comparable to HAP there are no further studies considering it as a HAP or analyzing this possibility. *P. ruminicola* is considered proteolytic, peptidolytic and deaminating bacteria. As its production was barely over the defined limit for HAP (300 nmol NH_3/min/mg protein), it would be necessary to analyze deeply their production and metabolism.

Enterococcus avium is a host-associated opportunistic pathogen focused on the intestinal microbiota and it has been isolated from humans, dogs, cats, and cows (Staley *et al*., 2014). Moreover, it was detected in feces from young cattle (Deviese *et al*., 1992), like in this study that it appeared from the ruminal fluid of heifers (M4). Even the NH_3 production was over 300 nmol NH_3/min/mg protein we could not consider it as a ruminal HAP, as it is not their natural habitat.

Eschenlauer *et al*. (2002) referred to *Acidaminococcus fermentans* as a HAP as it produced 10 times more NH_3 than the rest of the deaminating bacteria studied at that moment, between 168 – 285 nmol NH_3/min/mg protein. Although in Eschenlauer *et al*. (2002) study, *Acidaminococcus fermentans* did not reach the threshold to be considered HAP, in the present study, the production of NH_3 was even higher, 602 nmol NH_3/min/mg protein (Table 8).

Differences in microbial population found in the different groups (M1, M2, M3, M4) can be related to the differences in feed intake during different stages, as feed affects the rumen microbiome. 1–3-day old heifers showed *Streptococcus* as dominant species, young calves fed with whole milk presented more *Prevotella*, and in adult cattle the dominant population was *Bacteroidetes* (Ryu *et al*., 2022). Moreover, the microbial fecal composition of dairy heifers changes constantly during the first weeks of life (Malmuthuge *et al*., 2014), when diet is constantly changing and adapting to the animal requirements. This assumption matches the current work, as M4 was the one with the most variety of species compared to adult cow samples (M1, M2, M3), with mainly *Clostridium* species.

In addition, the modification of the cultivation method with Argon allowed to isolate *Acidaminococcus fermentans*, a strictly anaerobic bacterium.

Bacteriophages-HAP interaction

Few studies have reported ruminal bacteriophages. Ambrožič *et al*. (2001), Iverson and Millis, (1974), and Klieve *et al*. (1988) described bacteriophages isolated from the ruminal fluid that infect *Bifidobacterium ruminale, Fusobacterium necrophorum, Magnoovum eadii, Methanobrevibacter sp., Prevotella bryantii, Streptococcus bovis, Streptotoccous durans* and *Serratia spp.*, and most of them were in lysogenic state, representing a symbiosis between phages and hosts (Klieve *et al.,* 1996). Berg Miller *et al*., (2012) associated most of the ruminal fluid bacteriophages with the ruminal bacterial phylum *Firmicutes* and *Proteobacteria*.

Gilbert *et al*., (2017) reported the first complete genome of lytic bacteriophages related to *Bacteroides*, *Ruminococcus*, and *Streptococcus*, isolated from municipal sewage, bovine fecal waste material, and bovine ruminal fluid. The phages were classified as *Siphoviridae* and *Podoviridae*. At the same time, Anderson *et al*., (2017) observed that the most abundant phages and prophages in the ruminal fluid were from the *Mimiviridae*, *Myoviridae*, *Podoviridae* and *Siphoviridae* families, as also observed by TEM in the present study (Myoviridae, Podoviridae and Siphoviridae families).

In this chapter, isolated bacteria were from the genera *Clostridium*, *Streptococcus*, *Megasphaera*, *Prevotella*, and *Acidaminococcus*, in the phylum *Bacillota* and *Bacteroidota*. Only *Streptococcus* was susceptible to be host-related for the isolated phages (*Siphoviridae* and *Podoviridae* phage families). However, in this study, none of the isolated phages infected the HAP. The last experiment, *in vitro* cultivation of ruminal fluid with ruminal bacteriophages concentrate confirmed the absence of potential bacteriophages affecting the HAP as the NH_3 production were not significantly different from the control. There are existing phages infecting some of the isolated HAP, such as bacteriophage ATCC 8074-B1 for *Clostridium sporogenes* (Mayer *et al*., 2012). However, this phage was not isolated from ruminal fluid but from wastewater.

As bacteriophages affecting the isolated HAP were not find, the use of bacteriophages from a bank/collection to inoculate them into ruminal fluid and media for HAP in *in vitro* conditions could be a strategy to identify bacteriophages against HAP and to test them as a method to reduce rumen NH_3 production. Moreover, as the microbiota from the rumen tends to change with diet variations (Loor *et al*., 2016), ruminal fluid samples from different cows' diets could be useful to find different species of bacteriophages and HAP as well.

Conclusions

After isolating bacteria from dairy cows' ruminal fluid, we observed that *Clostridium sporogens*, *Terrisporobacter glycolicus* (*Clostridium glycolicum*), *Megasphaera elsdenii, Clostridium argentinense, Stretococcus sp., Prevotella ruminicola, and Acidaminococcus fermentans* presented an NH_3 production high enough to be considered as HAP (348 – 876 nmol NH_3/min/mg protein).

The effect of the cows' stage, directly related to the diet, influenced the variety of species we found in their ruminal fluid. *Clostridium* genera was the main HAP isolated from rumen samples of dairy cows and growing heifers. Other genera such as *Acidaminococcus* and *Terrisporobacter* were only present in dry cows, while *Megasphaera elsdenii* in heifers, suggesting physiological status or diet as factors influencing the presence of HAP.

The use of argon to improve anaerobiosis conditions allowed to isolate *Acidaminococcus fermentans*, a strict anaerobic bacterium, which produced 602 nmol NH_3/min/mg protein.

Although bacteriophages from the *Myoviridae*, *Podoviridae* and *Siphoviridae* families were identified in the ruminal fluid, none of them infected the obtained HAP. The *in vitro* assay did not show any interaction with the bacteriophages from dairy ruminal fluid. However, an *in vitro* assay using bacteriophages from a bank/collection against ruminal fluid may help to identify HAP phages evaluating NH_3 production.

References

Ambrožič, J., Ferme, D., Grabnar, M., Ravnikar, M., Avguštin, G. 2001. The bacteriophages of ruminal prevotellas. Folia Microbiol 46, 37–39. https://doi.org/10.1007/BF02825881.

Anderson, C. L., Sullivan, M. B., and Fernando, S. C. 2017. Dietary energy drives the dynamic response of bovine rumen viral communities. Microbiome 5:155. Doi: 10.1186/s40168-017-0374-3.

Bach, A., Calsamiglia, S., Stern, M.D. 2005 Nitrogen metabolism in the rumen. J Dairy Sci 88(13):E9–E21. 10.3168/jds.S0022-0302(05)73133-7.

Bach, A., Terré, M., Vidal, M. 2020. Symposium review: Decomposing efficiency of milk production and maximizing profit. Journal of Dairy Science, Volume 103, Issue 6, 2020, Pages 5709-5725, ISSN 0022-0302, https://doi.org/10.3168/jds.2019-17304.

Barron, M. 2022. Phage Therapy: Past, Present and Future. American Society for Microbiology. https://asm.org/Articles/2022/August/Phage-Therapy-Past,-Present-and-Future.

Bladen, H.A., Bryant, M.P., Doetsch, R.N. 1961. A study of bacterial species from the rumen which produce ammonia from protein 89eteorologi. Appl Microbiol. 9:175–80. 10.1128/am.9.2.175-180.1961.

Behera, S.N., Sharma, M., Aneja, V.P., Balasubramanian, R. 2013. Ammonia in the atmosphere: a review on emission sources, atmospheric chemistry and deposition on terrestrial bodies. Environ. Pollut. Res. Int. 20, 8092–8131.

Berg Miller, M. E., Yeoman, C. J., Chia, N., Tringe, S. G., Angly, F. E., Edwards, R. A., Flint, H.J., Lamed, R., Bayer, E.A., White, B.A. 2012. Phage-bacteria relationships and CRISPR elements revealed by a metagenomic survey of the rumen microbiome. Environ. Microbiol. 14, 207–227. Doi: 10.1111/j.1462-2920.2011.02593.x.

Calsamiglia, S., Ferret, A., Reynolds, C.K., Kristensen, N.B., van Vuuren, A.M. 2010. Strategies for optimizing nitrogen use by ruminants. Animal. Jul;4(7):1184-96. Doi: 10.1017/S1751731110000911.

Chen, G., Russell, J.B. 1989. More monensin-sensitive, ammonia-producing bacteria from the rumen. Appl Environ Microbiol. 55:1052–7. 10.1128/aem.55.5.1052-1057.1989.

Devriese, L.A., Laurier, L., De Herdt, P., Haesebrouck, F. 1992. Enterococcal and streptococcal species isolated from faeces of calves, young cattle and dairy cows. J Appl Bacteriol. 1992 Jan;72(1):29-31. Doi: 10.1111/j.1365-2672.1992.tb04877.x.

Dewanckele, L., Vlaeminck, B., Fievez, V. 2019. Sharpea azabuensis: a ruminal bacterium that produces trans-11 intermediates from linoleic and linolenic acid. Microbiology. 165. 10.1099/mic.0.000811.

Eschenlauer, S.C.P., McKain, N., Walker, N.D., McEwan, N.R., Newbold, C.J., Wallace, R.J. 2002. Ammonia production by ruminal microorganisms and enumeration, isolation, and characterization of bacteria capable of growth on peptides and amino acids from the sheep rumen. Appl Environ Microb 68(10):4925–4931. https:// doi.org/10.1128/aem.68.10.4925-4931.2002.

Firkins, J.L., Yu, Z., Morrison, M., 2007. Ruminal nitrogen metabolism: perspectives for integration of microbiology and nutrition for dairy. J. Dairy Sci. 90, 1–16.

Flythe, M.D., Andries, K. 2009. The effects of monensin on amino acid catabolizing bacteria isolated from the Boer goat rumen. Small Ruminant Res 81(2–3):178–181. https://doi.org/10.1016/j.smallrumres.2008.12.004.

Flythe, M.D., Harlow, B.E., Aiken, G.E., Gellin, G.L., Kagan, I.A., Pappas, J. 2017. "Inhibition of Growth and Ammonia Production of Ruminal HAP by Chinook or Galena Hops after Long-Term Storage" *Fermentation* 3, no. 4: 68. https://doi.org/10.3390/fermentation3040068.

Geishauer, T., Lihart, N., Neidl, A., Reimann, A. 2012. Factors associated with ruminal pH at herd level. J. Dairy Sci. 95, 4556-4567. https://doi.org/10.3168/jds.2012-5380.

Gerber, P. J. 2020. Nitrogen emissions along global livestock supply chains. Nature Food, 1(7), 437–446. https://doi.org/10.1038/s43016-020-0113-y.

Gilbert, R.A., Kelly, W.J., Altermann, E., Leahy, S.C., Minchin, C., Ouwerkerk, D., Klieve, A.V. 2017. Toward Understanding Phage:Host Interactions in the Rumen; Complete Genome Sequences of Lytic Phages Infecting Rumen Bacteria. Front. Microbiol. 8:2340. Doi: 10.3389/fmicb.2017.02340.

Gilbert, R.A., Townsend, E.M., Crew, K.S., Hitch, T.C.A., Friedersdorff, J.C.A., Creevey, C.J., Pope, P.B., Ouwerkerk, D., Jameson, E. 2020. Rumen Virus Populations: Technological Advances Enhancing Current Understanding. Front. Microbiol. 11:450. Doi: 10.3389/fmicb.2020.00450.

Harlow, B., Bryant, R., Cohen, S., O'Connell, S., Flythe, M. 2016. Degradation of spent craft brewer's yeast by caprine rumen HAP. Lett. Appl. Microbiol, 63. https://doi.org/10.1111/lam.12623.

Hartinger, T., Gresner, N., Südekum, K.H. 2018. Does intra-ruminal nitrogen recycling waste valuable resources? A review of major players and their manipulation. J Animal Sci Biotechnol 9, 33. https://doi.org/10.1186/s40104-018-0249-x.

Iverson, W.G., Millis, N.F. 1976. Characterization of Streptococcus bovis bacteriophages. Can J Microbiol. 1976 Jun;22(6):847-52. Doi: 10.1139/m76-122. PMID: 58706.

Klieve, A.V., Bain, P.A., Yokoyama, M.T., Ouwerkerk, D., Forster, R.J., Turner, A.F. 2004. Bacteriophages that infect the cellulolytic ruminal bacterium Ruminococcus albus AR67. Lett Appl Microbiol. 2004;38(4):333-8. Doi: 10.1111/j.1472-765x.2004.01493.x.

Klieve, A.V., Bauchop, T. 1988. Morphological diversity of ruminal bacteriophages from sheep and cattle. Appl. Environ. Microbiol. Vol 54, No. 6. https://doi.org/10.1128/aem.54.6.1637-1641.1988.

Klieve, A. V., Swain, R. A., and Nolan, J. V. 1996. Bacteriophages in the rumen: types presents, population size, and implications for the efficiency of feed utilization. Aust. Soc. Anim. Prod. 2, 92–94.

Kropinski, A.M., Mazzocco, A., Waddell, T.E., Lingohr, E., Johnson, R.P. 2009. Enumeration of bacteriophages by double agar overlay plaque assay. In Bacteriophages. Methods in Molecular Biology; Clokie, M.R., Kropinski, A.M., Eds.; Humana Press: Totowa, NJ, USA, 2009; Volume 501, pp. 69–76.

Kanuparthy A., Challa, T., Meegada, S., Siddamreddy, S., Muppidi, V. 2020. Staphylococcus warneri: Skin Commensal and a Rare Cause of Urinary Tract Infection. Cureus. 2020 May 28;12(5):e8337. Doi: 10.7759/cureus.8337. PMID: 32494545; PMCID: PMC7263002.

Loor, J.J., Elolimy, A.A., McCann, J.C. 2016. Dietary impacts on rumen microbiota in beef and dairy production, Animal Frontiers, Volume 6, Issue 3, July 2016, Pages 22–29, https://doi.org/10.2527/af.2016-0030.

Luo, J., Jiang, M., Xiong, J., Li, J., Zhang, X., Wei, H., Yu, J. 2018. Exploring a phage-based real-time PCR assay for diagnosing Acinetobacter baumannii bloodstream infections with high sensitivity, Analytica Chimica Acta, Volume 1044, Pages 147-153, ISSN 0003-2670, https://doi.org/10.1016/j.aca.2018.09.038.

Malmuthuge, N., Griebel, P.J., Guan, L.L. 2014. Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the gastrointestinal tracts of preweaned calves. Appl Environ Microbiol. Mar;80(6):2021-8. Doi: 10.1128/AEM.03864-13.

Mayer, M.J., Gasson, M.J., Narbad, A. 2012. Genomic sequence of bacteriophage ATCC 8074-B1 and activity of its endolysin and engineered variants against Clostridium sporogenes. Appl Environ Microbiol. 2012 May;78(10):3685-92. Doi: 10.1128/AEM.07884-11.

Millet, S., Aluwe, M., Van den Broeke, A., Leen, F., De Boever, J., De Campeneere, S. 2018. Review: Pork production with maximal nitrogen efficiency. Animal, 12, pp. 1060-1067.

Moodley, A., Kot, W., Nälgård, S., Jakociune, D., Neve, H., Hansen, L.H., Guardabassi, L., Vogensen, F.K. 2019. Isolation and characterization of bacteriophages active against methicillin-resistant Staphylococcus pseudintermedius. Res Vet Sci. 2019 Feb; 122:81-85. Doi: 10.1016/j.rvsc.2018.11.008.

Naureen, Z., Malacarne, D., Anpilogov, K., Dautaj, A., Camilleri, G., Cecchin, S., Bressan, S., Casadei, A., Albion, E., Sorrentino, E., Beccari, T., Dundar, M., Bertelli, M. 2020. Comparison between American and European legislation in the therapeutical and alimentary bacteriophage usage. Acta Biomed. Nov 9;91(13-S):e2020023. Doi: 10.23750/abm.v91i13-S.10815.

Paster, B. J., J. B. Russell, C. M. J. Yang, J. M. Chow, C. R. Woese, and R. Tanner. 1993. Phylogeny of the ammonia-producing ruminal bacteria Peptostreptococcus anaerobius, Clostridium sticklandii, and Clostridium aminophilum sp. Nov. Int. J. Syst. Bacteriol. 43:107–110.

Prosser, JI., Hink, L., Gubry-Rangin, C., Nicol, G.W. 2020. Nitrous oxide production by ammonia oxidizers: Physiological diversity, niche differentiation and potential mitigation strategies. In Global Change Biology (Vol. 26, Issue 1, pp. 103–118). Blackwell Publishing Ltd. https://doi.org/10.1111/gcb.14877.

Ripple, W.J., Smith, P., Haberl, H., Montzka, S.A., Mcalpine, C., Boucher, D.H. 2014. Ruminants, climate change and climate policy. Nat. Clim. Chang. 4, 2–5.

Ryu, C.H., Bang, H.T., Lee, S., Kim, B., Baek, Y.C. 2022. Effects of Feed Composition in Different Growth Stages on Rumen Fermentation and Microbial Diversity of Hanwoo Steers. Animals 2022, 12, 2606. https:// doi.org/10.3390/ani12192606.

Shen, J., Yu, Z., Zhu, W. 2018. Insights into the Populations of Proteolytic and Amino Acid-Fermenting Bacteria from Microbiota Analysis Using In Vitro Enrichment Cultures. Curr Microbiol 75, 1543–1550. https://doi.org/10.1007/s00284-018-1558-1.

Singh, B.K., Bardgett, R.D., Smith, P., Reay, D.S. 2010. Microorganisms and climate change: terrestrial feedbacks and mitigation options. Nat. Rev. Microbiol. 8, 779–790.

Staley, C., Dunny, G.M., Sadowsky, M.J. 2014. Chapter Four – Environmental and Animal-Associated Enterococci, Editor(s): Sima Sariaslani, Geoffrey M. Gadd, Advances in Applied Microbiology, Academic Press, Volume 87, 2014, Pages 147-186, ISSN 0065-2164, ISBN 9780128002612, https://doi.org/10.1016/B978-0-12-800261-2.00004-9.

Taban, B.M., Halkman, H.B., Halkman, A.K. 2014. Microflora of the intestine. Biology of the Enterococcus spp. Encyclopedia of Food Microbiology (Second Edition), 2014. Pages 652-657. https://doi.org/10.1016/B978-0-12-384730-0.00211-1.

Tan, P., Liu, H., Zhao, J., Gu, X., Wei, X., Zhang, X., Ma, N., Johnston, L.J., Bai, Y., Zhang, W., Nie, C., Ma, X. 2021. Amino acids metabolism by rumen microorganisms: Nutrition and ecology strategies to reduce nitrogen emissions from the inside to the outside, Science of The Total Environment, Volume 800, 149596, ISSN 0048-9697, https://doi.org/10.1016/j.scitotenv.2021.149596.

Tharaka, R.K.C., Doddapaneni, T.K. 2021. Chapter 39 – Thermochemical and biochemical treatment strategies for resource recovery from agri-food industry wastes, Editor(s): Rajeev Bhat, Valorization of Agri-Food Wastes and By-Products, Academic Press, 2021, Pages 787-807, ISBN 9780128240441, https://doi.org/10.1016/B978-0-12-824044-1.00007-6.

Ti, C., Xia, L., Chang, S. X., Yan, X. 2019. Potential for mitigating global agricultural ammonia emission: A meta-analysis. Environmental Pollution, 245, 141–148. https://doi.org/10.1016/j.envpol.2018.10.124.

Uwizeye, A., de Boer, I.J.M., Opio, C.I., Schulte, R.P.O., Falcucci, A., Tempio, G., Teillard, F., Casu, F., Rulli, M., Galloway, J.N., Leip, A., Erisman, J.W., Robinson, T.P., Steinfeld, H., Gerber, P.J. 2020. Nitrogen emissions along global livestock supply chains. Nature Food, 1(7), 437–446. https://doi.org/10.1038/s43016-020-0113-y.

Yun, M.J., Yoon, S., Lee, Y.J. 2020. Monitoring and Characteristics of Major Mastitis Pathogens from Bulk Tank Milk in Korea. Animals (Basel). Sep 2;10(9):1562. Doi: 10.3390/ani10091562.

Zhang, N., Bai, Z., Ledgard, S., Luo, J., Ma, L. 2021. Ammonia mitigation effects from the cow housing and manure storage chain on the nitrogen and carbon footprints of a typical dairy farm system on the North China Plain. Journal of Cleaner Production, 280. https://doi.org/10.1016/j.jclepro.2020.124465.

Chapter 7 – Acidification and solar drying of manure-based digestate to produce improved fertilizing products

CRediT authorship contribution statement

L. Morey: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **B. Fernández:** Conceptualization, Methodology, Validation, Investigation, Writing – review & editing, Supervision. **L. Tey:** Support on Formal analysis, Investigation. **C. Biel:** Conceptualization, Methodology, Support on Formal analysis, Investigation, Writing – review & editing. **A. Robles-Aguilar:** Validation, Support on Formal analysis, Investigation, Writing – review & editing. **E. Meers:** Writing – review & editing, Supervision, Funding acquisition. **J. Soler:** Resources. **R. Porta:** Resources. **M. Cots:** Resources. **V. Riau:** Conceptualization, Methodology, Validation, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Abstract

The increase in energy and fertilizer consumption makes it necessary to develop sustainable alternatives for agriculture. AD and digestates appeared to be suitable options. However, untreated digestates still have high water content and can increase greenhouse gas emissions during storage and land application. In this study, manure-derived digestate and solid fraction of digestate after separation were treated with a novel solar drying technology to reduce their water content, combined with acidification to reduce the gaseous emissions. The acidified digestate and acidified solid fraction of digestate recovered more TN and TAN than their respective non-acidified products (1.5 – 1.3 times for TN; 14 times for TAN). NH_3 and CH_4 emissions were reduced up to 94% and 72% respectively, compared to the non-acidified ones, while N_2O increased more than 3 times. DD and DAD can be labeled as NPK organic fertilizer according to the European regulation, and the dried solid fraction and the improved dried acidified solid fraction can be labeled as N or P organic fertilizer. Moreover, plant tests showed that N concentrations in fresh lettuce leaves were within the EU limit with all products in all cases. However, zinc concentration appeared to be a limitation in some of the products as their concentration exceeded the European legal limits.

The content of this chapter has been published as Morey, L., Fernández, B., Tey, L., Biel, C., Robles-Aguilar, A., Meers, E., Soler, J., Porta, R., Cots, M., Riau, V. 2023. Acidification and solar drying of manure-based digestate to produce improved fertilizing products. Journal of Environmental Management. Volume 336, 15 June 2023, 117664.

https://doi.org/10.1016/j.jenvman.2023.117664.

Introduction

The consumption of fertilizers in Europe increased 6.9% for nitrogen and 21.9% for phosphorous since 2010 (Eurostat, 2022) and it has been a tendency since the past decades (Liu *et al*., 2015). Therefore, the recovery of biobased fertilizers from animal manure to partially replace synthetic mineral fertilizers should be considered a key strategy to move towards a more sustainable agriculture. In this regard, AD is a valuable process to treat livestock manure since, apart from the generation of biogas as a renewable energy source, it allows to recycle nutrients from the derived digestate as fertilizer (Barzee *et al*., 2019; Horta and Carneiro, 2022), besides some other alternative valorizations (ethanol production, nutrient-enriched microalgae, or membrane concentration of specific nutrients). However, depending on the agricultural practices during their storage and land application, digestates could release volatile organic compounds and gaseous emissions, and generate water eutrophication (Battista and Bolzonella, 2019; Salamat *et al*., 2020). Therefore, further processing of digestates to improve their fertilizer efficiency and minimize emissions should be studied.

Different technologies have been proven to handle digestate or recover their nutrients. Mechanical separation is usually the first step, splitting digestate into concentrated and clarified fractions that are further treated afterwards. The clarified or "liquid" fraction can go through membrane separation that concentrates nitrogen and phosphorus compounds using a selective barrier (Sengupta *et al*., 2015) or through the stripping process to recover ammonium as ammonium sulfate (Laureni *et al*., 2012) and ammonium nitrate (Sigurnjak *et al*., 2019). The concentrated or "solid" fraction can also be treated by composting to stabilize the OM (Cáceres *et al*., 2018) or drying to reduce its water content (Angouria-Tsorochidou, 2022). Focusing on drying technologies, many installations have found that drying the digestate or the solid fraction of digestate is an economically viable approach because the end-product would strongly reduce its volume, being more suitable for exportation due to a reduction of transportation and storage costs (Lebuf *et al*., 2012) (Salamat, 2020).

Opposite to conventional thermal dryers, solar dryers have been used as a traditional method for food preservation (Jairaj *et al*., 2009). Moreover, in countries with high solar radiation, it is an energetically sustainable method (Ndukwu *et al*., 2018). In 2005, the "Institute of Heat Engineering, Warsaw University of Technology" developed the concept of solar dryers for wastewater where the energy use is considerably lower than in other drying facilities (Krawczyk *et al*., 2011). Conventional driers (convective drying, conductive drying, fry drying) require a specific energy consumption between 700 – 1400 kWh per ton of evaporated water, while solar dryers 30 – 200 kWh per ton of evaporated water when they are combined with heated floors (Salamat *et al*., 2020). Nowadays, solar drying for sewage sludge is a reality, with companies

developing solar drying treatment systems at full-scale. Recently, Battista and Bolzonella (2019) have studied the solar drying of digested slurry to recover ammonium sulfate using a solar dryer with a greenhouse configuration at the laboratory scale, promoting the use of solar energy in the treatment of waste. However, solar dryers have not been tested yet at full scale to produce organic fertilizers from manure-derived digestates.

One of the risks when drying nitrogen-rich digestates is the volatilization of nitrogen as NH_3 (Stambaskhy, 2013). In this sense, an interesting approach to reduce NH_3 emissions is using acidic agents to shift the acid-base equilibrium to ammonium (NH_4^+) (Fangueiro *et al*., 2015). Prenafeta *et al*. (2021) studied the combination of acidification with the solar drying of fresh pig slurry to control NH_3 and GHG emissions on a pilot scale. Recently, Dalby *et al*. (2022) reported a decrease in CH_4 emissions by 63-99% during the management of pig slurry after acidification at pH ≈ 5.5. However, there are no references to studies that measure emissions at a bigger scale and, at the same time, aim to produce fertilizers from digested manure as a valorization or post-treatment technology.

This work aimed to assess the efficiency of a nutrient recovery process of manure-based digestate that combined the acidification, solar drying, and final addition of the N-poor liquid fraction obtained after the stripping of the liquid fraction of digestate in the production of more sustainable organic fertilizers. The study focuses on reducing water content, conservation of nutrients, and reduction of GHG and acidifying emissions, followed by a pot phytotoxicity test and a comparison to the current European Fertilizers Regulation to determine the viability of the final products.

Materials and methods

Fertilizers production at a semi-industrial scale

The production of digestate-derived fertilizers (Figure 25) was done in two periods. First, a set of four products were obtained: dried digestate (DD), dried acidified digestate (DAD), dried solid fraction of digestate (DSF), and dried acidified solid fraction of digestate (DASF). Second, after the first results, an improved trial was performed to produce a new dried acidified solid fraction of digestate (DASF2) and a dried mixture (DM) of the acidified solid fraction of digestate (ASF) with a secondary stream with a low nitrogen content (stripped liquid fraction; SLF), coming from a stripping process, in a ratio ASF:SLF of 3:1 (wet mass).

All fertilizers were produced by duplicate in a semi-industrial solar drying plant, with two greenhouse-type solar dryers (Figure 26), beside a biogas plant (Vila-sana, Lleida, Spain) where the digestate was generated. This biogas plant treated pig manure and agro-industrial wastes (on a yearly average, pig manure 41%, sewage sludge mixture 50%, and others 9% of total incoming daily inflow) at 37ºC with a hydraulic retention time of 50 days. The sewage sludge mixture (on

a yearly average, slaughterhouse sewage sludge 30%, municipal sewage sludge 9%, dairy sewage sludge 6%, brewery sewage sludge 5%) was designed by the biogas plant operator considering that the concentration of heavy metals per each type of sludge was always below the limit defined by the Spanish regulation about the use of sewage sludge in the agricultural sector (RD 1310/1990).

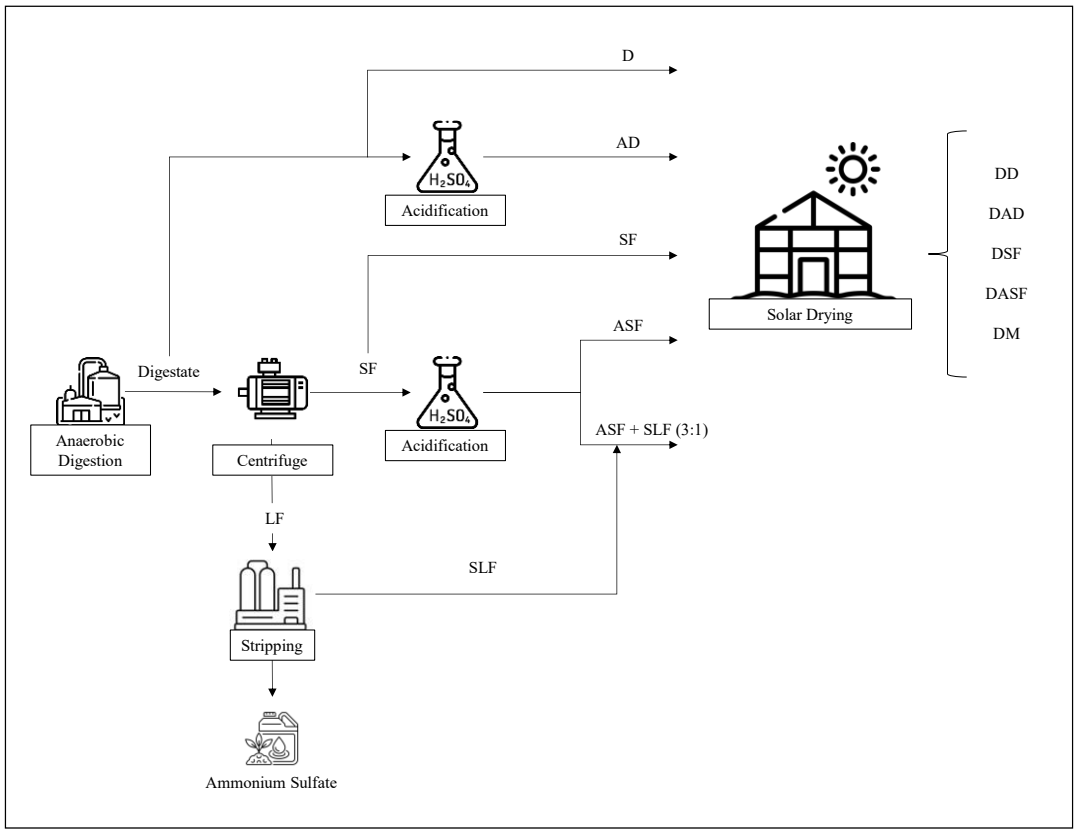


Figure 25. Diagram with all the digestate-derived fertilizers produced. Abbreviations: D, digestate; AD, acidified digestate; C, centrifuge; SF, solid fraction of digestate; LF, liquid fraction of digestate; ASF, acidified solid fraction; SLF, poor-nitrogen stream after LF stripping process; DD, dried digestate; DAD, dried acidified digestate; DSF, dried solid fraction; DASF, dried acidified solid fraction; DM, dried mixture of ASF and SLF.

Figure 26. Greenhouse solar dryer and turning machine in the semi-industrial installation.

The production of the digestate-derived fertilizers was performed from mid-April to mid-September (same period in years 2020 and 2021), with an ambient temperature ranging from 4.9ºC to 40.1ºC in 2020, -0.3ºC to 41.2ºC in 2021, and an annual mean solar radiation of 16.3 MJ/m^2 (Servei Meteorològic de Catalunya, 2021). First, a concentrated stream or solid fraction (SF) was produced from digestate by a solid-to-liquid separation process (centrifugation plus a previous addition of a polyelectrolyte-type flocculant and antifoam agent).

The acidification of digestate or SF was done once before drying, adding a solution of sulfuric acid (richness 50%; doses of 0.028 g-sulfuric/kg) till a pH of 5.5-6.0 according to the best available techniques to avoid NH_3 emissions (Satonja *et al*., 2017). This process was controlled by a digital pH controller and the pump was adding sulfuric acid solution into a screw that fed the solar dryer until the desired pH was reached. Two drying lines were available; each comprised one solar dryer, one air blower, and one biofilter. Each greenhouse solar dryer had a working area of 468 m^2 (total area of 625 m^2; length 80 m; width 7.8 m; height 3.7 m), divided into two subareas of 234 m^2 each (length 30 m) to dry per duplicate each product simultaneously. A turning machine (Figure 30) distributed the material evenly and prevented crusting (maximum thickness of 40 cm). A natural airflow (mean flow of 2.8 m/s) was sucked from inside the dryer with a blower (operating 10 h/d with 100% of its maximum flow) that directed it to the biofilter (height 5.1 m; diameter 2.35 m), filled with pine bark and mature compost (ratio 10:1, in volume). The front door of the greenhouse driers remained open to promote natural airflow convection. The drying process was operated in batch mode (4,000 kg per batch for digestate or AD and 3,600 kg per batch for SF or ASF).

The clarified fraction of digestate or liquid fraction (LF) after centrifugation was submitted to a stripping process (Figure 25), followed by an absorption process to recover NH_3 as ammonium sulfate (AS; richness 17%). In addition to AS, a low nitrogen concentration stream or SLF was obtained after stripping. This stream was blended with ASF, in a ratio ASF:SLF =3:1, to produce a dried mixture (DM) to improve the nutrient (mainly nitrogen) concentration of DASF and valorize SLF or secondary stream.

During the drying process, the temperature of the air and products was registered, reaching temperatures up to 58ºC in the materials (Figure 27). Samples of the initial, intermediate (once per week), and final materials, as well as gaseous emissions (NH_3, CH_4, N_2O), were collected. For the sampling, the drying area (234 m^2) was divided into two equivalent zones (117 m^2) where two sampling points were fixed. In these points, representative samples of the corresponding materials were taken within a 10-20 cm depth. These two sampling points per drying zone were also used to collect the gaseous emissions. The effect of acidification was assessed by comparing the acidified and non-acidified products in terms of (i) recovered TN, recovered TAN, TC, TP, and TK; (ii) the reduction of GHG and NH_3 emissions; and (iii) phytotoxicity of the products.

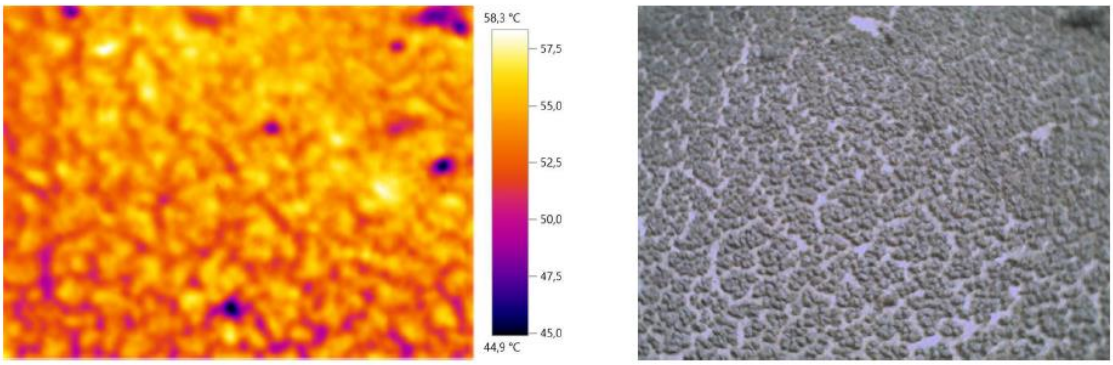


Figure 27. Thermographical image of the digestate being dried inside the greenhouse solar dryer (3rd August 2020).

Gaseous emissions

Gaseous samples were taken inside the semi-industrial solar dryers using a dynamic chamber (dimensions 1030 x 530 x 250 mm; Odournet GmbH, Germany), placed directly above the material and equipped with a pump that ensured a laminar airflow (Prenafeta-Boldú *et al*., 2015; Torrellas *et al*., 2018). The temperature inside each solar dryer was registered while sampling. All samples were collected in duplicate.

The samples (30 mL) for the determination of the GHG (GHG: CH_4, N_2O, CO_2) were collected in 12.5 mL vacutainer-type tubes (Labco Ltd., Buckinghamshire, UK) per duplicate. This measurement was done using a gas chromatograph (Thermo Trace 2000, Thermo Finnigan Scientific) equipped with a flame ionization detector and an electron capture detector (model 7820A, Agilent). Results were expressed as ppm at 25ºC.

The measurement of the NH_3 concentration was also done in situ with a portable sensor (model GX-6000, RKI Instruments; sensitivity range of 0.5 to 150 ppm v/v) equipped with an electrochemical detector, directly connected to the outflow of the dynamic chamber. When the sensor was not available, samples for NH_3 determination were taken by bubbling 3 L of air (outflow of the dynamic chamber) into 20 mL acid solution (sulfuric acid 10%) tubes, which were analyzed by UV-VIS spectrometry (Hach Lange DR2800) according to the NIOSH 6015 method (Eller and Cassinelli, 1994). Results were expressed as ppm at 25ºC.

The GHG and NH_3 emission flux (mg/m^2h) at 25ºC was calculated by multiplying the corresponding concentration, after converting gas concentrations from ppm to mg/m^3 by equation (2) (National Research Council US, 2001) at 25 ºC, by the hood internal flux (30 m^3/m^2 h). The cumulated gas emission after 21 days was calculated by the trapezoidal method of integration (Levy *et al*., 2017; Dalby *et al*., 2022).

Eq.2 $$ppm_{NTP} = (mg/m^3) \bullet (24.46/MW) \bullet (760/P) \bullet (T/298)$$

Where P, sampling site pressure (mm Hg); T, sampling site temperature (K); MW, molar weight (g/mol); NTP, normal temperature and pressure.

The measured GHG were expressed as equivalent of CO_2 (tCO_2eq) using 28 mg_{CO2}/mg_{CH4} and 265 mg_{CO2}/mg_{N2O} as conversion factors (Myhre *et al*., 2013). The emissions of CO_2 were not considered to calculate the total CO_2 equivalent due to the livestock CO_2 is net-zero (Gavrilova, 2019).

Phytotoxicity assay

A pot experiment with seedlings of lettuce (variety Maravilla) was made to test the potential phytotoxicity and fertilizing effect of the dried products compared to a commercial soluble fertilizer (Agrolution). Each pot had a volume of 250 cm^3. The substrate used in the experiment was a mixture of peat and perlite in a 1:1 ratio. The watering was done daily to keep the proper moisture content. Four aqueous extracts (ratio 1:10, in volume) of the products DD, DAD, DSF, and DASF were prepared, mixing 100 g of material and 1 L of distilled water and letting stand for 6 hours before filtering. Each extract was diluted 85%, 75%, 50%, 25%, and 0% with distilled water. The pH, conductivity, TAN, and NO_3-N were measured in each extract. After observing the high NH_4^+ concentrations in the DAD extracts, it was decided to apply more diluted extracts only for this product: 90%, 80%, 70%, and 50% dilution. Twelve applications of 20 mL of each extract and dilution per plant were added to the corresponding pot during the growing period (n=20). The plants were grown in a heated greenhouse (IRTA Cabrils, Spain) for 63 days avoiding temperatures below 5ºC. After harvesting, the plants' fresh weight of 10 replicates was measured, and consecutively, plants were dried at 60 °C for 72 hours until constant weight. Dry biomass was ground to particles <2 mm.

Physic-chemical characterization of fertilizers and plant analyses

Samples of initial raw materials, as well as intermediate and final samples of dried fertilizers, were characterized. The Standard Methods (APHA, 2005) were followed to measure conductivity (method 2510); pH (method 4500-H^+); total ammonia-nitrogen (TAN; method 4500-NH3-C), determined using a Buchi distillation unit (BUCHI Labortechnik GmbH, The Netherlands); and TS and VS (method 2540G) of all samples. The content of TC and TN was measured in all samples using an elemental analyzer LECO® (Leco Corporation, Michigan, USA) (ISO-13878, 1998). In addition, the products at the beginning and end of the drying process were characterized by their content of total phosphorus (TP, expressed as P_2O_5), total potassium (TK, expressed as K_2O), TOC, and heavy metals. The content of TP and TK was determined by optical emission spectrometry ICP-OES (US Department of Agriculture, 2018).

Plants from the growth assessment tests were subjected to microwave digestion (CEM MARS 6, USA). The nutrient content and metal concentration in the samples were analyzed by ICP-OES.

The TC and TN contents were analyzed using the CN analyzer (Skalar Analytical BV, the Netherlands).

Data analysis and statistics

After analyzing several digestates from the biogas plant (Table 10) and comparing them with data from the literature (Table 11), it became clear that the wide variability in the characteristics of the digestate depends on the feedstock of the anaerobic digesters. The same analysis were performed with SF and SLF. Thus, given the variability of initial materials shown in Table 10, recovery indexes were calculated in this study using the mean value of each characterizing parameter for every drying batch (initial vs. final material per batch). Then, the nutrient recovery efficiency and the reduction of gaseous emissions were compared between the acidified products with their non-acidified equivalents.

One way ANOVA was used as a statistical analysis to determine the significance of the effect of acidification on the digestate and solid fraction of digestate emissions (total accumulated CH_4, NH_3 and N_2O in gCO_{2eq}/m^2), with a confidence value of 95%, using IBM SPSS as statistical software (SPSS Inc., Chicago, USA).

For the plant biomass growth, Tuckey mean separation was performed via one way ANOVA both for dry weight and wet weight.

Table 10. Characteristics of digestates (D), concentrated fractions of digestates (SF), clarified fraction of digestates (LF), sampled in 2 years of experimentation. Abbreviations: TC, total carbon; TN, total nitrogen; $N\text{-}NH_4^+$, ammonium; TS, total solids; VS, volatile solids, TP, total phosphorus; TK, total potassium.

	TC	TN	$N\text{-}NH_4^+$	TS	VS	TP	TK
Dry weight	%	%	%	%	%	%	%
Digestate D	33	10	6.9	5.3	64	2.9	1.4
	-	22	14	2.9	65	2.7	1.3
	19	5.2	7.2	6.9	64	-	-
	32	5.9	8.1	6.5	65	-	-
	33	6.2	7.3	6.9	64	-	-
	34	6.1	7.1	7.1	65	-	-
	34	5.5	7.1	7.1	67	-	-
	37	5.7	7.8	6.6	62	-	-
	35	5.9	7.4	6.8	64	-	-
	34	5.2	6.7	7.7	65	-	-
	34	5.2	7.1	7.1	62	-	-
	30	11	7.5	7.0	60	-	-
	30	19	13	4.0	92	-	-
	29	11	7.5	7.1	54	-	-
	24	15	10	5.2	58	-	-
	29	11	7.3	7.3	56	-	-
	28	38	7.8	6.9	58	-	-
	29	37	7.2	7.4	59	-	-
	29	10	7.0	7.4	59	-	-
	28	10	7.1	7.3	56	-	-
Average D	29	12	8.1	-	63	6.4	1.6
Std.dev.	7.7	9.7	1.9	-	7.5	0.3	0.1

Table 10. Continuation. Characteristics of digestates (D), concentrated fractions of digestates (SF), clarified fraction of digestates (LF), sampled in 2 years of experimentation.

	TC	TN	$N\text{-}NH_4^+$	TS	VS	TP	TK
	34	38	32	1.0	66	-	-
	31	17	13	3.5	56	-	-
Liquid fraction of digestate (LF)	32	24	20	2.3	61	-	-
	31	19	19	3.3	63	-	-
	48	59	43	0.9	65	-	-
Average LF	35	32	25	-	62	-	-
Std.dev.	6.4	16	11	-	3.4	-	-
	34	5.2	1.9	24	58	2.7	0.3
	31	5.9	1.8	25	59	2.7	0.4
	58	6.2	4.2	14	57	-	-
Solid fraction of digestate (SF)	59	5.8	2.0	20	99	-	-
	29	5.2	1.9	29	56	-	-
	31	5.1	2.1	24	57	-	-
	33	5.1	2.2	25	57	-	-
	33	5.1	2.2	25	57	-	-
Average SF	38	5.4	2.3	-	62	2.7	0.3
Std.dev.	11	0.4	0.8	-	14	0.0	0.1

Table 11. Comparison of digestates from several AD plants from literature. Abbreviations: LF (liquid fraction); SF (solid fraction); n.a. (not available).

Material	Influent of the biogas plant	TS (%)	VS (%TS)	TC (%TS)	TN (%TS)	TAN (%TS)	TP (%TS)	TK (%TS)	Reference
D	41% pig manure – 59% agro-industrial wastes	6.5 ±1.2	63±7.5	29±7.7	12±9.7	8.1±1.9	2.8±0.1	1.4±0.1	this study
LF	41% pig manure – 59% agro-industrial wastes	2.6±1.0	62±3.4	35±6.4	32±16	25±11	n.a.	n.a.	this study
SF	41% pig manure – 59% agro-industrial wastes	23±4.1	62±14	38±11	5.5±0.4	2.3±0.7	2.7±0.1	0.3±0.1	this study
D	Agricultural wastes+ cattle manure + poultry manure	4.3	50	n.a.	11	6.4	n.a.	n.a.	Torrisi et al., 2021
D	Pig manure + energetic crops	4.4	70	40	12	8.8	1.0	2.7	Jimenez et al., 2020
D	Average of pig slurry, cow slurry and energetic crops	6.1	n.a.	n.a.	8.1	4.9	1.6	n.a.	Tambone et al., 2017
LF	Sugar beet pulp	3.8	86	15	8.5	4.9	0.1	5.6	Chuda & Zieminski, 2021
LF	Cattle manure	8.8	39	n.a.	8.4	4.4	4.3	11	Valentinuzzi et al., 2020
LF	Pig slurry, cow slurry and energetic crops	4.5	n.a.	n.a.	9.8	6.0	1.6	n.a.	Tambone et al., 2017
SF	Pig slurry, cow slurry and energetic crops	21	n.a.	n.a.	2.9	1.0	1.3	n.a.	Tambone et al., 2017
SF	Wastewater treatment sludge	22	52	27	4.0	1.1	2.0	0.2	Jimenez et al., 2020

Results

Production performance at semi-industrial scale

Nutrient recovery

The drying process at semi-industrial scale was fulfilled between 21 and 35 days, and the emission comparison was done on the 21st day to compare the emissions of all of the products (Figure 27), depending on the products, being the drying of digestates faster than SF or ASF. This can be explained because the same amount of product occupied less volume in the case of digestate (with a 10 cm layer deposited) compared to the SF or ASF (40 cm layer along the semi-industrial dryer). All the products were dried to attain a TS of 90%, except for the mixture that was dried until a lower TS content (close to 50%) to be easily applied in fields and fulfilled the farmers' requirement. The average chemical composition of the raw materials (Digestate, SF, and SLF) and dried products are shown in Table 12.

Acidified products (DAD and DASF) recovered more TN and TAN than the non-acidified products (DD and DSF): 1.5 and 1.3 times, respectively, for TN; 14 times each one for TAN. Concerning potassium, both acidified products recovered more potassium than the non-acidified ones (1.7 times for DAD and 1.4 times for DASF). The recovered TP and TC of DAD production were lower than in its non-acidified relative product (0.68 and 0.86 times, respectively). On the other hand, the recovery of TP and TC of DASF production was higher than in the DSF production (1.13 and 1.07 times, respectively).

Table 12. Physic-chemical characterization of digestate-derived products. Abbreviations: % wm, wet mass base; % TS, dry mass base; n.d., not determined; D, raw digestate; DD, dried digestate; DSF, dried solid fraction of digestate; DAD, dried acidified digestate; DASF, DASF2, dried acidified solid fraction of digestate; DM, dried mixture of ASF and SLF. Note: *mean values.

		Raw materials			Semi-industrial scale				Improvement trial	
Parameter	units	D*	SF*	SLF*	DD	DSF	DAD	DASF	DM	DASF2
Conductivity	mS/cm	27	n.d.	n.d.	4.6	1.8	20.4	2.9	6.0	6.6
pH	-	8.3	n.d.	n.d.	7.8	7.5	5.7	8.0	6.4	7.9
TS	%wm	6.5	23	2.9	90	91	89	85	47	83
VS	%TS	63	62	63	60	60	59	61	39	57
P2O5	%TS	2.8	2.7	n.d.	6.3	6.9	3.8	6.9	6.1	5.1
K2O	%TS	1.4	0.3	n.d.	1.6	0.4	2.5	0.5	0.4	0.5
TC/TN		0.6	6.9	n.d.	6.7	10	3.8	8.4	8.4	7.8
TC	%TS	29	38	n.d.	33	34	25	32	52	25
TOC	%TC	-	n.d.	n.d.	100	100	100	100	33	100
TN	%TS	48	5.5	12	4.9	3.4	6.5	3.8	6.2	3.2
TAN	%TS	8.1	2.3	7.4	0.3	0.3	3.5	3.3	0.8	1.1
Cd	mg/kgTS	<0.5	n.d.	n.d.	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Cu	mg/kgTS	167	n.d.	n.d.	149	152	95	173	134	115
Cr (VI)	mg/kgTS	<0.5	n.d.	n.d.	<0.5	<0.5	<0.5	<0.5	n.d.	n.d.
Hg	mg/kgTS	<0.4	n.d.	n.d.	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4
Ni	mg/kgTS	19	n.d.	n.d.	16	12	17	13	21	16
Pb	mg/kgTS	7.2	n.d.	n.d.	7.7	6.9	<5	7.4	8.1	6.9
Zn	mg/kgTS	884	n.d.	n.d.	738	780	512	891	816	663

Gaseous emissions

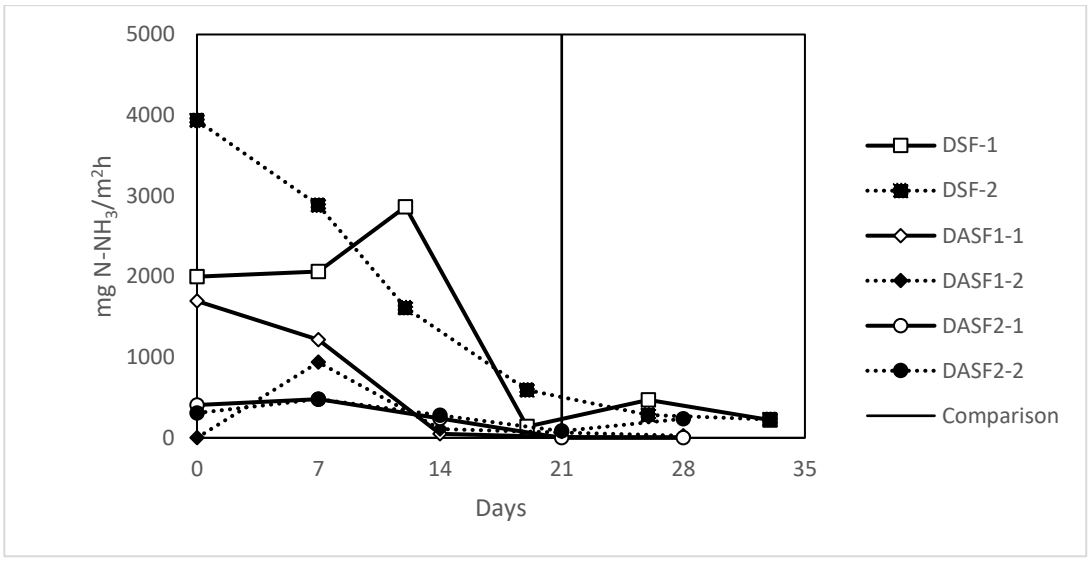
Emissions of NH_3 and GHG were measured for each fertilizer production, under batch mode, in the semi-industrial driers. Table 13 summarizes the total cumulative emissions (NH_3, CH_4, and N_2O).

Table 13. Total cumulative emission of GHGs and NH_3 after 21 days. n = 2. Note: Sum of net CH_4, N_2O, expressed in CO_2 eq units/m^2. (*) Comparison between DSF and DASF2 or DM. Abbreviations: DD, dried digestate; DSF, dried concentrated fraction of digestate; DAD, dried and acidified digestate; DASF, dried and acidified solid fraction of digestate 2020; DASF2, dried and acidified solid fraction of digestate 2021; DM, dried mixture of ASF:SLF; non-AP, non-acidified product.

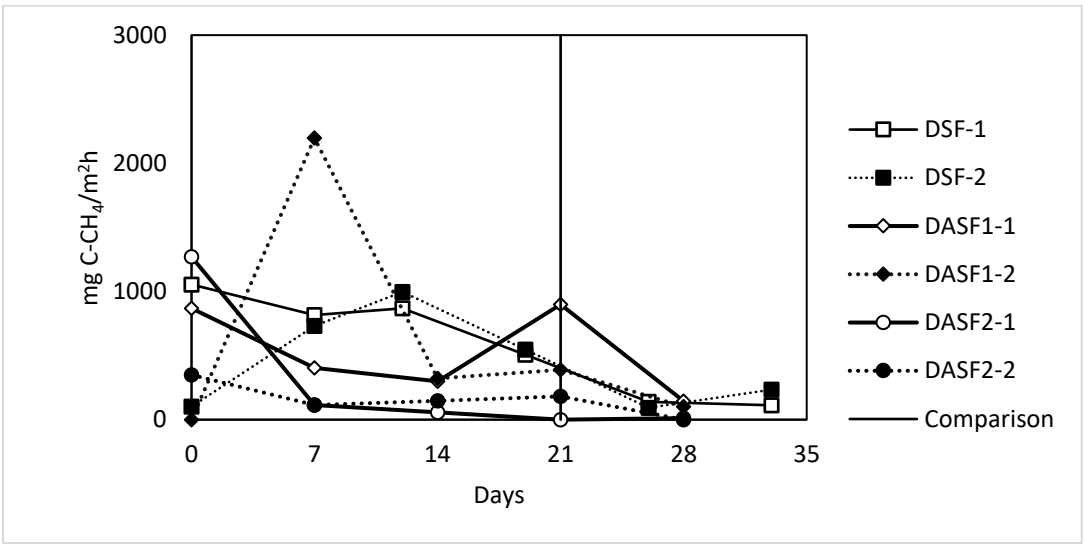
Total emitted	Units	DD	DAD	DSF	DASF	DASF2*	DM*
CH_4	kg CO_2 eq/m^2	2.4	1.08	11	12	4.8	5.7
CH_4	p value	0.383		0.779		<0.001	0.122
N_2O	kg CO_2 eq/m^2	8.2	59	35	124	17	25
N_2O	p value	0.007		0.005		0.218	0.524
NH_3	g NH^3/m^2	389	25	1169	327	223	235
NH_3	p value	<0.001		<0.001		<0.001	<0.001

NH_3 emissions decreased during the drying of acidified products (Figure 28). The total cumulative emission of NH_3 were 25 and 327 g-NH_3/m^2 for DAD and DASF, respectively, while these emissions for the corresponding non-acidified products were 389 and 1169 g-NH_3/m^2 for DD and DSF, respectively. This means that acidification reduced significatively the total cumulative NH_3 emission by 94% drying digestate and 72% drying the solid fraction of digestate (SF).

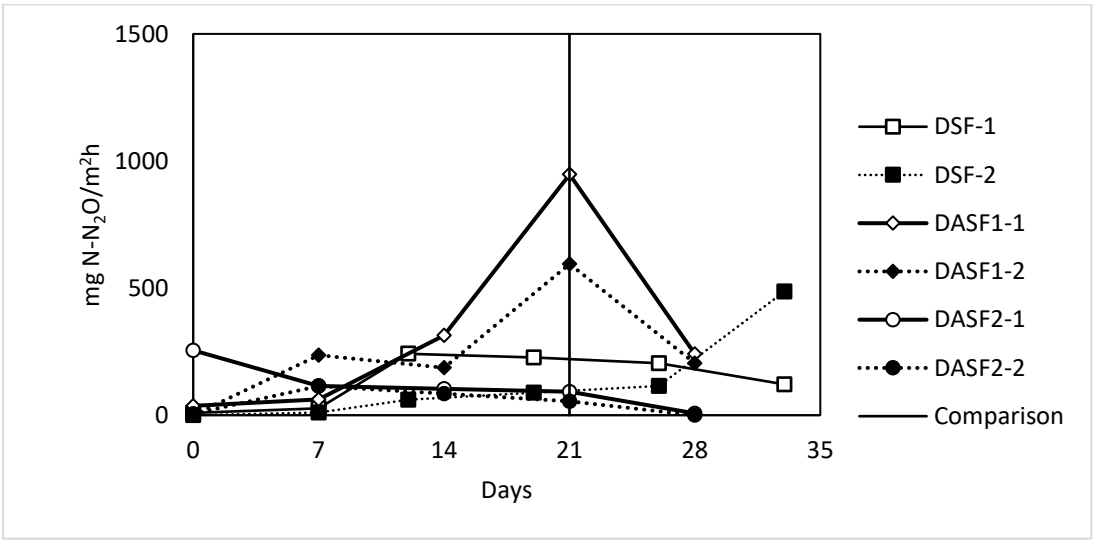
Regarding DASF compared to DSF, acidification caused an increment of N_2O and CH_4 emissions, 251% ($p < 0.05$), and 5.8% ($p > 0.05$) respectively. Concerning DAD compared to DD, there is an increase in N_2O, 620% ($p < 0.05$), and a reduction of CH_4 of 54% ($p > 0.05$).



a)



b)



c)

Figure 28. Profile of emission fluxes (mg/m^{2}h), measured during the solar drying of solid fraction (SF) derived products. (a) N-NH_3. (b) C- CH_4. (c) N-N_2O. Symbols: Solid line: first replicate; dashed line: second replicate; square: DSF; diamond: DASF; circle: DASF2; vertical line: 21 days were selected to calculate the total accumulated emissions per product for comparison purposes.

Improved production of dried fertilizing products

Based on previous results, the turning machine was modified to improve the aeration, by installing perpendicular flippers to increase the removal of the crust, and the sulfuric acid addition was improved by changing the dosing point, which allowed a better pH measurement. Within these changes, a second batch for drying acidified solid fraction of digestate (ASF) was performed, producing DASF2 (Tables 12 and 13). Based on a comparison with DSF, a clear reduction of all emissions was shown, including N_2O and CH_4. As can be seen in Figure 27, the effect of acidification is clearly improved, not only related to the reduction of emissions but also reducing the deviation between replicates. For DASF2, emission was reduced significatively by 81% and 57% for NH_3 and CH_4, and a reduction ($p > 0.05$) of 53% for N_2O, compared to DSF.

Another improved approach was the mixture of the ASF with a waste stream coming from the stripping plant from the AD facility (SLF). The stream ASF was the best candidate to produce the mixture as it contained the greatest TP content and showed a clear decrement in NH_3 emissions while drying. The idea of producing the mixture was to enhance the fertilizing value of the ASF by using the SLF as an additional nitrogen source. In addition, a secondary objective was to valorize the high volume of SLF, that remained after the stripping of LF. Therefore, ASF:SLF was blended in a 3:1 ratio and dried. As a result, the TN content of the dried mixture was higher than the TN content of the DASF (Table 12); however, the conductivity of the mixture also increased as the SLF still had a high concentration of salts. Regarding gaseous emissions, compared to DSF, the NH_3 emission was reduced by 80% ($p < 0.05$), 28% for N_2O ($p > 0.05$), and 50% ($p > 0.05$) for CH_4.

Phytotoxicity effect of the recovered products

A plant growth test was performed with extracts of the recovered products (not the mixtures) to observe possible phytotoxic effects in the juvenile stage of lettuce. The extracts applied had different values of pH, EC, and NH_4^+ (Table 14), which significantly affected the lettuce's fresh and dry weight (Table 15).

The maximum concentration applied for DAD in this growth test was 50%. Still, the $NH4^+$ content in the extracts was much higher in this treatment than with the other products. Consequently, N concentrations analyzed in plants treated with DAD (>6 gN/100 gDW-plant) (Table 19) were in the high or toxic range of N in plant tissue as defined by Marschner (2011).

Table 14. pH, EC, and NH_4^+ concentrations measured in the extracts. Abbreviations: n.a., not available.

	Extracts														
Treatment	pH					EC (mS/cm)					NH_4^+ (mg/L)				
Dilution	85	75	50	25	0	85	75	50	25	0	85	75	50	25	0
DD	n.a.	7.8	7.8	7.8	7.8	n.a.	2.2	3.2	4.4	5.7	n.a.	21.9	34.5	49.3	64.1
DSF	n.a.	8	7.9	7.9	7.9	n.a.	1.4	1.7	2.1	2.5	n.a.	24.0	42.0	63.0	83.4
DASF	n.a.	8	7.9	7.9	8	n.a.	1.5	1.7	2.0	2.5	n.a.	9.4	19.0	27.9	39.6
Dilution	90	80	70	50	/	90	80	70	50	/	90	80	70	50	/
DAD	7.4	7.1	6.9	6.6	n.a.	3.1	5.3	7.3	11.1	n.a.	216	256	282	284	n.a.

Table 15. Lettuce fresh and dry weight (g plant^{-1}), total nitrogen (TN), total carbon (TC), and leaves Zn concentrations. Note: *Plants were dead. Different letters show the statistical differences by Tuckey mean separation.

Treatment	DD				DAD				DSF				DASF			
Dilution	75	50	25	0	90	80	70	50	75	50	25	0	75	50	25	0
								g plant -1								
Fresh weight	2.5 (bcde)	3.9 (a)	3.5 (ab)	3.3 (abc)	2.0 (de)	1.9 (de)	1.7 (d)	†*	2.9 (abcd)	2.3 (cde)	3.4 (ab)	3.5 (ab)	1.6 I	2.1 (de)	1.6 (e)	2.3 (cde)
Dry weight	0.3 (abcd)	0.3 (a)	0.3 (ab)	0.3 (abc)	0.2 (bcd)	0.2 (d)	0.2 (cd)	†	0.3 (abc)	0.2 (abcd)	0.3 (abcd)	0.3 (abc)	0.2 (abcd)	0.2 (abcd)	0.2 (cd)	0.3 (abc)
								% TS								
TN	3	3.2	3.5	3.9	5.5	6.2	6.3	†	2.7	3.2	4.1	4.3	2.9	3	3.4	3.4
C	38	37	37	35	37	37	37	†	35	37	38	37	39	37	38	38
								mg/kgTS								
Zn	128	161	109	102	129	154	121	†	56	75	171	165	72	108	107	102

The dose applied did not have a significant effect on the plant biomass within each treatment (p value < 0.05); however, non-acidified products led to higher biomass in general than the acidified counterparts. Furthermore, plants treated with > 30% extracts (70% dilution) of DAD died, indicating a growth inhibition due to the high EC and NH_4^+ concentrations found in this treatment.

After observing Zn concentrations higher than regulation limits (Zn > 800 mg/kg) in the chemical analyses of the DASF, the concentrations of metals in plant tissue were analyzed, to measure if they were within the average values for lettuce. The results showed no significant differences in Zn uptake in plants treated with DASF compared to plants treated with the non-acidified DSF. Despite the Zn concentrations found in edible tissues of lettuce (Table 15) being higher than the expected value (Li *et al*, 2016), the EU regulation does not include a plant tissue limit concentration approach. Other elements analyzed in the tissue of lettuce were within the normal range, and elements such as Cd, Co, Cr, Cu, Ni, and Pb were less than the detection limit.

Discussion

Solar drying combined with acidification improved the efficiency and sustainability of the process at pilot scale

Acidified products DAD and DASF recovered 1.5 and 1.3 times more TN and 14 and 14 times more TAN than the non-acidified products, respectively. The obtained values of nutrient recovery after acidification are higher than the ones reported previously by Liu *et al*., (2019), who recovered up to 6.2 times more TAN in a thermal process with acidified digestate at pH 6.5 (1 point higher than in this study) than the same digestate without acidification. This increment could be explained by the microbial activity regarding ammonification and/or the hydrolysis of organic nitrogen (Moure Abelenda *et al*., 2021), and as it appeared to be, the lower the pH, the higher the N recovery, especially TAN, as NH_4^+ (ionic form) is higher than NH_3 (non-ionic/volatile form).

NH_3 emissions dramatically decreased during the drying of acidified products, 94% and 72% for DAD and DASF respectively, and a major decrease of 81% and 80% for DASF2 and DM compared to DSF. This is in line with the data reported by Wagner *et al*. (2021), which determined a decrease of 89-96% in NH_3 emissions during the field application of acidified pig slurry (pH 6), compared to the non-acidified one. Eihe *et al*. (2019) obtained a reduction of up to 90% of NH_3 emission with acidified digestate (pH 6.5) during its application to the field, compared to the non-acidified digestate.

Despite the turning machine that removed the upper crust during the drying for the solid fraction products, total or partial nitrification conditions could be attained in the lower part of the material. The peak of N_2O emissions was placed when the TS content of the composite sample was > 30%. Moreover, acidification increased the available TAN to be transformed by nitrogen oxidizing

bacteria (Cytryn *et al.*, 2012) to produce N_2O, NO_2 or NO_3 than in the non-acidified product. Nevertheless, after the improvements were done in the turning machine and the acidification, N_2O emission was reduced compared to the previous system (the reduction of N_2O emission was 53%) due to the reduction of crust in the surface. In the case of digestate drying, the formation of the crust, which was not removed, and the enhancement of nitrogen oxidizing bacteria due to acidification could explain the high emission levels of N_2O.

In addition, acidification is supposed to inhibit methanogenesis by decreasing the activity of methanogenic bacteria (Pantelopopulos and Aronsson, 2021). However, the mixture of solid fraction with acid did not differ widely from the non-acidified. This can be related to a non-optimized acidification procedure because after the system was improved, CH_4 emissions decreased compared to the non-acidified product (Table 13). When the acidification system was improved for DASF2, CH_4 emission was reduced considerably, up to 57%.

There are few references regarding digestate acidification, but some regarding the acidification of fresh manure. Miranda *et al*., 2021 presented a 98% reduction in CH_4 emissions by acidifying weekly cattle slurry to maintain a pH of 5.5 during storage. Im *et al*. (2020) demonstrated a 70% reduction of CH_4 emissions during pig slurry storage acidifying to pH 6.5. Pantelopoulos and Aronsson (2021) showed a 50% reduction with a liquid fraction of pig slurry acidified to 5.9. All of them with similar CH_4 reduction values as those obtained by the acidification of digestate in this study (73%).

Biobased products obtained as candidates for the European fertilizer legislation and RENURE criteria

These products were compared with the European criteria to determine their potential application as organic fertilizers, following Regulation 2019/1009 of the European Parliament and of the Council (Regulation EU 2019/1009), describing the requirements and the limitations on the application of organic fertilizers. In general, NPK solid organic fertilizers must have a concentration of TN > 1% TS, P_2O_5 > 1% TS, K_2O > 1% TS, NPK > 4% TS, and TOC > 15% TS. For a solid organic fertilizer declaring only a primary nutrient, the required concentrations are TN > 2.5% TS, or P_2O_5 > 2% TS, or K_2O > 2% TS.

According to the EU regulation, regarding nutrients (NPK) criteria, only DD and DAD, with nutrient concentrations higher than the minimum established by the legislation, are suitable to get the CE label to be marketed in the European Union. Each country's regulation will determine the limitations to be used in the field. Considering only N or P fertilizer criteria, all the products were appropriate to be applied as fertilizers.

However, DASF and DM would be rejected due to their concentration of Zn which was above the threshold of 800 mg/kg. This is explained by the high concentrations of Zn found in the pig

slurry used as a major substrate in the AD plant of this study: zinc is normally used as an additive to stimulate animal growth and prevent diseases (Alburquerque *et al*., 2012). The presence of heavy metals in digestate is associated more to the manure than the agricultural wastes, related to the supplements for commercial feedstuff to promote optimum nutrient supply and growth (Demirel *et al*., 2013)

Also, the dried products were compared with future guidelines for recovered nitrogen from manure (RENURE) products that define the quality and/or handling rules that a processed manure material should comply to be classified as a "*substance fully or partially derived from livestock manure through processing that can be used in areas with water pollution by nitrogen following otherwise identical provisions applied to nitrogen containing chemical fertilizers as defined in the Nitrates Directive (91/676/EEC), while ensuring the achievement of the Nitrates Direct've's objective and providing adequate agronomic benefits to enhance plant growth*" (Huygens *et al.*, 2020). It is necessary to fulfill one of the following criteria: (i) TOC:TN $0 \leq p \leq 3$ or (ii) TAN:TN > 90%, where p is the evaluated product. Moreover, copper and zinc concentrations must be < 300 and < 800 mg/kg, respectively. In agreement with the RENURE criteria, only DASF was close to fulfill the requirements with a TAN: TN ratio of 89%. However, Zinc concentration was higher than the established limit of 800 mg/kg, as well. Reuland *et al*., (2021) analyzed 2800 data from unpublished and literature digestate and liquid fraction analyses for RENURE criteria, and the results showed that liquid fraction is better suited to RENURE criteria than digestate, with compliance between 3 - 58%.

The plant tests showed that N concentrations in lettuce fresh leaves were within the EU limit (< 3,000 mg/kg fresh weight) with all products in all the cases, as the European Union establishes maximum permissible levels from 4,000 to 5,000 mg $N\text{-}NO_3^-$/kg fresh weight for the winter season (Commission Regulation 1258/2011). However, the N concentrations (> 6 gN/100g TS plant) (Table 15) analyzed in lettuce plants treated with DAD were in the high/toxic range of N in plant tissue as defined by Marschner (2011). Interestingly, even though Zn concentration in DASF was higher than law limits (> 800 mg/kg), no significant differences in Zn uptake were observed compared to plants treated with the non-acidified DSF in the higher doses. Also, Zn concentrations found in edible tissues of lettuce were higher than the expected value of 7.9 mg/kg TS (Li *et al*, 2016), but no limit is established in the regulation.

Although more studies need to be conducted to study the full growing cycle of lettuces, we can preliminarily advance that all concentrations of DD, DSF and DASF can be applied for growing lettuces, as the dose applied did not have an effect on the plant biomass and the concentration of heavy metals and NO_3^- are below the regulation limits. However, for DAD, concentrations higher than 30% can have a negative effect on the plants.

Conclusions

DASF and DAD recovered 1.3 and 1.5 times more TN than the non-acidified DSF and DD counterparts and 14 times more TAN. Moreover, the acidified products reduced the NH_3 emissions by up to 94% and 72% for DAD and DASF, compared to the non-acidified ones. On the other hand, N_2O emissions increased 620% and 251% for DAD and DASF, compared to their non-acidified relatives,

DD and DAD fit the European regulation of fertilizers to be labeled as solid organic NPK fertilizers, and DSF, DASF, and DM could be labeled as Nitrogen or Phosphorus solid organic fertilizers. DASF and DM had a concentration of Zn superior to limit established by the regulation, but no significant differences in Zn concentration appeared in the plant leaves. Moreover, the dose of application did not have a significant effect on plant biomass. Finally, plant tests showed that N concentrations in fresh lettuce leaves were within the EU limit with all products in all the cases.

References

Alburquerque, J.A., De la Fuente, C., Ferrer-Costa, A., Carrasco, L., Cegarra, J., Abad, M., Bernal, M.P. 2012. Assessment of the fertiliser potential of digestates from farm and agroindustrial residues. Biomass and Bioenergy. Volume 40. https://doi.org/10.1016/j.biombioe.2012.02.018.

Angouria-Tsorochidou, E., Seghetta, M., Trémier, A., Thomsen, M. 2022. Life cycle assessment of digestate post-treatment and utilization Sci Total Environ. 815. p. 152764. https://doi.org/10.1016/j.scitotenv.2021.152764

Anuari de dades meteorològiques 2020. Servei Meteorològic de Catalunya. https://www.meteo.cat/wpweb/climatologia/serveis-i-dades-climatiques/series-climatiques-historiques/. Accessed in April 2021.

APHA. 2005. Standard Methods for the Examination of Water and Wastewater. [2]2nd edition. ISBN: 0875530478.

Barzee, TJ., Edalati, A., El-Mashad, H., Wang, D., Scow, K., Zhang, R. 2019. Digestate Biofertilizers Support Similar or Higher Tomato Yields and Quality Than Mineral Fertilizer in a Subsurface Drip Fertigation System. Front. Sustain. Food Syst. 3:58. https://doi.org/10.3389/fsufs.2019.00058.

Battista, F., Bolzonella, D. 2019. Exploitation of Solar Energy for Ammonium Sulfate Recovery from Anaerobic Digestate of Different Origin. Waste and Biomass Valorization. doi:10.1007/s12649-019-00597-x

Cáceres, R., Malińska, K., Marfà, O. 2018. Nitrification within composting: A review. Waste Management, Volume 72, Pages 119-137, ISSN 0956-053X, https://doi.org/10.1016/j.wasman.2017.10.049.

Commission Regulation (EU) No 1258/2011 of 2 December 2011 amending Regulation (EC) No 1881/2006 as regards maximum levels for nitrates in foodstuffs Text with EEA relevance.

Council Directive 91/676/EEC of 12 December 1991 concerning the protection of waters against pollution caused by nitrates from agricultural sources.

Cytryn, E., Levkovitch, I., Negreanu, Y., Dowd, S., Frenk, S., Silber, A. 2012. Impact of short-term acidification on nitrification and nitrifying bacterial community dynamics in soilless cultivation media. Applied and environmental microbiology. 78(18). 6576–6582. https://doi.org/10.1128/AEM.01545-12.

Dalby, F.R., Guldberg, L.B., Feilberg, A., Kofoed, M.V.W. 2022. Reducing greenhouse gas emissions from pig slurry by acidification with organic and inorganic acids. PLoS ONE 17(5):e0267693. https://doi.org/10.1371/journal.pone.0267693.

Demirel, B., Göl, N.P. & Onay, T.T. 2013. Evaluation of heavy metal content in digestate from batch anaerobic co-digestion of sunflower hulls and poultry manure. J Mater Cycles Waste Manag 15, 242–246. Doi: 10.1007/s10163-012-0107-4.

Eihe, P., Verberen, L.L., Grinfelden, I., Pilecka, J., Schapazidou, V., Grinberga, L. 2019. The effect of acidification of pig slurry digestate applied on winter rapeseed on the ammonia emission reduction. IOP Conf. Ser.: Earth Environ. Sci. 390 012043. doi: 10.1088/1755-1315/390/1/012043.

Eurostat. 2022. Agri-environmental indicator – mineral fertilizer consumption. https://ec.europa.119eteoroltat/statistics-explained/index.php?title=Agri-environmental_indicator_-_mineral_fertiliser_consumption#Analysis_at_EU_level (Accessed April 2022)

.Fangueiro, D., Hjorth, M., Gioelli, F. 2015. Acidification of animal slurry–a review. J Environ Manage.149: 46–56. pmid:25463570. DOI: 10.1016/j.jenvman.2014.10.001.

Gavrilova, O., Leip, A., Dong, H., MacDonald, J.D., Gomez, C.A.B., Amon, B., et al. 2019. Emissions from livestock and manure management. Refinement to the 2006 IPCC Guidelines for National Greenhouse Gas Inventories: Agriculture, Forestry and Other Land Use. IPCC; 2019.

Horta, C., Carneiro, J.P. 2022. Use of Digestate as Organic Amendment and Source of Nitrogen to Vegetable Crops. Appl. Sci. 12. 248; https://doi.org/10.3390/app12010248.

Huygens, D., Orveillon, G., Lugato, E., Tavazzi, S., Comero, S., Jones, A., Gawlik, B., Saveyn, H. 2020. Technical proposals for the safe use of processed manure above the threshold established for Nitrate Vulnerable Zones by the Nitrates Directive (91/676/EEC). EUR 30363 EN. Publications Office of the European Union. Luxembourg. ISBN 978-92-76-21540-0. doi:10.2760/984729. JRC121636.

Im, S., Mostafa, A., Shin, S.R., Kim, D.H. 2020. Combination of H2SO4-acidification and temperature-decrease for eco-friendly storage of pig slurry J. Hazard. Mater. 399. Article 123063. doi:10.1016/j.jhazmat.2020.123063.

ISO 13878:1998. Soil quality — Determination of total nitrogen content by dry combustion ("elemental analysis").

Jairaj, K.S., Singh, S.P., Srikant, K. 2009. A review of solar dryers developed for grape drying. Solar Energy, 83, 1698-1712. https://doi.org/10.1016/j.solener.2009.06.008.

Krawczyk, P., Badyda, K. 2011. Modeling of thermal and flow processes in a solar wastewater sludge dryer with supplementary heat supply from external sources. Journal of Power Technologies 91 (1) 37–40.

Laureni M., Palatsi J., Llovera M., Bonmatí A. 2013. Influence of pig slurry characteristics on ammonia stripping efficiencies and quality of the recovered ammonium-sulfate solution. J. Chem. Technol. Biotechnol. 88:1654–1662. doi: 10.1002/jctb.4016.

Lebuf, V., Accoe, F., Vaneeckhaute, C., Meers, E., Michels, E., Ghekiere, G. 2012. Nutrient recovery from digestates: techniques and end-products. In Proceedings of IV International Symposium on Energy from Biomass and Waste, Venice.

Levy, P., Cowan, N.J., Van Oijen, M., Famulari, D. Drewer, J. Skiba, U. 2017. Estimation of cumulative fluxes of nitrous oxide: uncertainty in temporal upscaling and emission factors: Estimation of cumulative fluxes of nitrous oxide. European Journal of Soil Science. 68. doi:10.1111/ejss.12432.

Li, B., Wang, Y., Jiang, Y., Li, G., Cui, J. 2016. The accumulation and health risk of heavy metals in vegetables around a zinc smelter in northeastern China.Environ Sci Pollut Res Int23: 25114-25126. doi: 10.1007/s11356-016-7342-5.

Liu, J., De Neergaard, A., Jensen, L.S. 2019. Increased retention of available nitrogen during thermal drying of solids of digested sewage sludge and manure by acid and zeolite addition. Waste Management. Volume 100. Pages 306-317. ISSN 0956-053X. https://doi.org/10.1016/j.wasman.2019.09.019.

Liu, Y., Pan, X., Li, J. 2015. A 1961–2010 record of fertilizer use, pesticide application and cereal yields: a review. Agronomy for Sustainable Development 35 (1), pp.83-93. ff10.1007/s13593- 014-0259-9ff.

Marschner, H. (Ed.). 2011. Marsch’er's mineral nutrition of higher plants. Academic press.

Miranda, C., Soares, A.S., Coelho, AC., Trinidade, H., Teixeira, C.A. 2021. Environmental implications of stored cattle slurry treatment with sulphuric acid and biochar: a life cycle assessment approach. Environ. Res. 194. p. 110640. doi:10.1016/j.envres.110640.

Moure Abelenda, A., Semple, K.T., Lag-Brotons, A.J., Herbert, B.M.J., Aggidis, G., Aiouache, F. 2021. Impact of sulphuric. hydrochloric. nitric. and lactic acids in the preparation of

a blend of agro-industrial digestate and wood ash to produce a novel fertiliser J. Environ. Chem. Eng. 9. Article 105021. doi:10.1016/j.jece.2020.105021.

Myhre, G., Shindell, D., Bréon, F.M., Collins, W., Fuglestvedt, J., Huang, J., Koch, D., Lamarque, J.F.,Lee, D., Mendoza, B., Nakajima, T., Robock, A., Stephens, G., Takemura, T., Zhang, H. 2013. Anthropogenic and natural radiative forcing. In Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press. pp. 659-740. doi:10.1017/CBO9781107415324.018.

National Institute for Occupational Safety and Health. Eller, P. M., Cassinelli. M. E. & United States. 1994. NIOSH manual of analytical methods. Washington. DC: United States Government Printing Office.

National Research Council (US) Subcommittee on Acute Exposure Guideline Levels. Standing Operating Procedures for Developing Acute Exposure Guideline Levels for Hazardous Chemicals. Washington (DC): National Academies Press (US); 2001. PMID: 25057561.

Ndukwu, M.C., Bennamoun, L., Abam, F. I. 2018. Experience of solar drying in Africa: Presentation of designs, operations and models. Food Engineering Reviews, December 2018, Volume 10, Issue 4, pp 211–244. doi:10.1007/s12393-018-9181-2.

Pantelopoulos, A., Aronsson, H. 2021. Two-stage separation and acidification of pig slurry-Nutrient separation efficiency and agronomical implications. J. Environ. Manag.. 280 (15). p. 111653. https://doi.org/10.1016/j.jenvman.2020.111653.

Prenafeta-Boldú, F.X., Viñas, M., Noguerol, J., Guivernau, M., Tey, L., Bonmatí, A. 2015. Influence of deep litter on gaseous emissions and bed microbial composition in a dairy farm. (Körner I. ed.) pp. 360-363. TuTech Innovation GmbH. Hamburg. Germany.

Prenafeta-Boldú, F.X., Fernández, B., Viñas, M., Noguerol, J., Soler, J., Illa, J. 2021. Combined Acidification and Solar Drying of Pig Slurries for Nutrient Recovery and Controlled Atmospheric Emissions. Agronomy 2021, 11, 222. https://doi.org/10.3390/agronomy11020222.

Real Decreto 1310/1990 de 29 de octubre por el que se regula la utilización de los lodos de depuración en el sector agrario. https://www.boe.es/eli/es/rd/1990/10/29/1310.

Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC.) No 1069/2009 and (EC.) No 1107/2009 and repealing Regulation (EC.) No 2003/2003.

Reuland, G., Sigurnjak, I., Dekker, H., Michels, E., Meers, E. 2021. The Potential of Digestate and the Liquid Fraction of Digestate as Chemical Fertiliser Substitutes under the RENURE Criteria. Agronomy. 2021; 11(7):1374. https://doi.org/10.3390/agronomy11071374.

Salamat, R., Scaar, H., Weigler, F., Berg, W., Mellmann, J. 2020. Drying of biogas digestate: A review with a focus on available drying techniques. drying kinetics. and gaseous emission behavior. Drying Technology. doi: 10.1080/07373937.2020.1781879.

Santonja, G.G., Georgitzikis, K., Scalet, B.M., Montobbio, P., Roudier, S., Sancho, L.D. 2017. Best Available Techniques (BAT) Reference Document for the Intensive Rearing of Poultry or Pigs; EUR 28674 EN; doi:10.2760/020485.

Sengupta, S., Nawaz, T., Beaudry, J. 2015. Nitrogen and Phosphorus Recovery from Wastewater. Curr Pollution Rep 1. 155–166. https://doi.org/10.1007/s40726-015-0013-1.

Sigurnjak, I., Brienza, C., Snauwaert, E., De Dobbelaere, A., De Mey, J., Vaneeckhaute, C., Michels, E., Schoumans, O., Adani, F., Meers, E. 2019. Production and performance of bio-based mineral fertilizers from agricultural waste using ammonia (stripping-)scrubbing technology. Waste Management, Volume 89, 2019, Pages 265-274, ISSN 0956-053X. https://doi.org/10.1016/j.wasman.2019.03.043.

Stambasky, J. 2013. Opinion on the Digestate Drying at Biogas Plants. European Biogas Association (EBA). Drying technology journal. Volume 40. https://doi.org/10.1080/07373937.2020.1781879.

Torrellas, M., Burgos, L., Tey, L., Noguerol, J., Riau, V., Palatsi, J., Antón, A., Flotats, X., Bonmatí, A. 2018. Different approaches to assess the environmental performance of a cow manure biogas plant. Atmospheric Environment 177. 203. doi: 10.1016/j.atmosenv.2018.01.023.

U.S. Department of Agriculture. 2018. Determination of metals by ICP-NS an ICP.OES (Optical Emission Spectrometry) [CLG-TM3.06]. Washington. DC: Food Safety and Inspection Service. Office of Public Health Science.

Wagner, C., Nyord, T., Vestergaard, A.V., Hafner, S.D., Pacholski, A.S. 2021. Acidification Effects on In Situ Ammonia Emissions and Cereal Yields Depending on Slurry Type and Application Method. Agriculture. 11(11):1053. https://doi.org/10.3390/agriculture11111053.

Chapter 8 – Germination and growth response of lettuce (*Lactuca sativa*) under greenhouse conditions fertilized by novel biobased fertilizing products derived from pig manure

CRediT authorship contribution statement

L. Morey: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **B. Fernández:** Conceptualization, Validation, Investigation, Writing – review & editing, Supervision. **C. Biel:** Conceptualization, Methodology, Support on Formal analysis, Investigation, Writing – review & editing. **A. Robles-Aguilar:** Validation, Support on Formal analysis, Investigation, Writing – review & editing. **E. Meers:** Writing – review & editing, Supervision, Funding acquisition. **V. Riau:** Conceptualization, Methodology, Validation, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Abstract

Substitution of synthetic mineral nitrogen fertilizers by biobased products derived from agro-wastes is a key strategy towards sustainable agriculture. In this study, pig manure-derived digestate, as well as its concentrated fraction, treated with a novel solar drying technology combined with acidification, were analyzed for toxicity in germination and pot trials with lettuce. Extracts of the products obtained in Chapter 8 (DD, DAD, DSF, DASF, DM1, DM3, DM6), diluted 25%, 50%, 75% and 100%, were applied in a seed germination test with lettuce seeds, with no detrimental effects excepts for product DAD and 0% dilution of DD and DM3 due to the immediately contact of high quantity of salts with the seeds. Moreover, DASF was able to generate a biostimulation effect on them. A pot experiment was performed afterwards, using lettuce (variety Maravilla), to test the growth and quality of the edible part of the plants. A dose of 150 kgN/ha was applied to all products. Fresh and dry weight, plant diameter, SPAD, and leachate were measured to determine the effect of the fertilizers on their production. Except for M6, all fertilizers produced a beneficial increase on the plant edible part, and nutrient recovery compared to the negative control (No-N) and fit the current legislations for heavy metals and potential toxic elements (PTE) in soil and plant. No differences in $N\text{-}NH_4^+$ leaching were found and DSF, DAD and DM3 reduced NO_3^- leaching ($p < 0.05$) compared to the control.

Introduction

Substitution of synthetic mineral fertilizers by products derived from manure has been regarded as a key strategy to integrate sustainable agriculture and livestock production (Luo *et al*., 2022). Manure appears to pose challenges in processing and application management, nonetheless there is a high potential for nutrient recovery while mitigating nutrients losses and GHG emissions (Vergote *et al*., 2020) from the livestock industry. Annually, 1.4 billion tons of manure are produced in Europe and UK (Köninger *et al*., 2021), and less than 10% (7.8%) of the total manure received additional treatment to produce more efficient fertilizing products, the rest is directly spread to agricultural fields (Buckwell and Nadeu, 2016).

Several technologies have been employed for manure processing, including AD, solid-liquid separation, membrane filtration and evaporation, which can lead to the production of biobased products with a higher ratio of plant-available N than the raw manure (Riva *et al*., 2016; Alburquerque *et al*., 2012a, b; Walsh *et al*., 2012). In a pig farm in Catalonia, Spain, a novel nutrient recovery cascade was developed to increase nutrient concentration in recovered products and reducing gaseous emissions at the same time (Chapter 7). The process proved the high efficiency of solar drying and acidification in reducing energy consumptions, transportation volume (and hence costs) and the greenhouse emissions.

The resulting biobased products have been analyzed in this study to prove their potential to partially substitute synthetic N fertilizer.

Phytotoxicity is a widely used parameter to evaluate the product application impact on crops and it represents an index of its overall ecotoxicological impact (Da Ros *et al*., 2018). The results can provide useful insights into the positive benefits of these products, thus helping in valorizing them.

Germination index (G_i) has been considered as one of the first parameters to analyze the phytotoxicity of the products (Anjum *et al*., 2018; Lencioni *et al*., 2016).

However, it has been shown that further analyses are needed in order to define the agronomic value of the products and simulate a more realistic scenario of how the novel products affect crops and horticultural plants. Therefore, in this study both G_i and plant growth experiments were performed to analyze the phytotoxicity and fertilizer efficiency of the recovered products.

This study aimed to (i) evaluate the fertilizer performance of the biobased products derived from pig manure digestate through germination and pot trials using lettuce as test plant, and (ii) compare the observed performance with criteria to be considered as organic fertilizers in the European Fertilizing Product Regulation. We hypothesized that (i) innovative manure processing increases the fertilizer value of the recovered biobased products compared to raw manure or

digestates, and (ii) the products can fit the current European legislation of fertilizers 2019/1009 of June 2019.

Material and methods

Fertilizers production

The products obtained from the pig farm, with a biogas plant for manure in situ valorization, were DD, DAD, DSF, DASF, and a mixture ASF and SLF in a ratio of ASF:SLF = 3:1 (DM1), in a semi-industrial scale solar dryer, described in Chapter 7.

Furthermore, two mixtures (DM3 and DM6) were produced mixing the ASF and SLF, both collected in the pig manure biogas plant, at different ratios ASF:SLF: 1:1 and 1:8 (in wet mass) for DM3 and DM6, respectively. These mixtures were directly dried in a greenhouse solar drier pilot plant (IRTA Torre Marimon, Caldes de Montbui, Spain) (Figure 29) that replicated the semi-industrial solar drying and biofiltration processes described in Chapter 7.

Figure 29. Greenhouse solar dryer pilot plant system.

The pilot-scale plant was operated between September 2021 and February 2022 for the mixture from pig farm, with an outside temperature ranging from 4.8 ºC to 32.9 ºC and a mean solar irradiation of 15.5 MJ/m^2 (Servei meteorologic de Catalunya, 2021).

The pilot plant consisted of four identical garden-type greenhouses acting as solar dryers, with an area of 0.92 m^2 each (length 1.80 m; width 0.51 m; height 0.51 m), connected to a biofilter to ensure a clean air release. Each material was spread by gravity (maximum thickness of 10 cm). The drying process was operated in batch mode with 30 liters per replicate. The biofilter was filled with crushed pine pieces and mature compost with a ratio of 10:1 (volume). Two temperature probes, to measure both temperatures inside and outside the solar dryer, and a relative humidity probe (connected to a data-logger system that registered data every 15 minutes) were used to measure both temperatures inside and outside the solar dryer. Also, the relative humidity

(RH) was monitored every 15 minutes. This probe was located at the middle of each dryer and controlled the functioning of the air blower, switching on the blower when if RH > 60%. The ambient and internal temperatures ranged 4.9-39 ºC and 6.7-65 ºC, respectively, and the mean relative humidity was 60%. The characterization of the final materials is described in Table 16.

Table 16. Chemical analyses of the dried products. Abbreviations: DD, dried digestate; DAD, dried acidified digestate; DSF, dried solid fraction of digestate; DASF, dried acidified solid fraction of digestate; DM1, mixture (ASF:SLF 3:1); DM3, mixture (ASF:SLF 1:1); DM6, mixture (ASF:SLF 1:8).

Parameter	DD	DSF	DAD	DASF	DM1	DM3	DM6
Conductivity (mS/cm)	4.6	1.8	20.4	2.9	6.0	11	6.7
pH	7.8	7.5	5.7	8.0	6.4	6.8	7.7
TS (%ww)	90	91	89	85	47	57	40
VS (%TS)	60	60	59	61	39	53	52
P2O5 (%TS)	6.3	6.9	3.8	6.9	6.1	2.8	5.1
K2O (%TS)	1.6	0.4	2.5	0.5	0.4	0.3	0.8
TC/TN	6.7	10	3.8	8.4	8.4	5.5	7.0
TC (%TS)	33	34	25	32	52	26	28
TOC (%TC)	100	100	100	100	63	100	89
TN (%TS)	4.9	3.4	6.5	3.8	6.2	4.7	4.0
TAN (%TS)	0.3	0.3	3.5	3.3	0.8	1.8	2.4
Cd (mg/kgTS)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Cu (mg/kgTS)	149	152	95	173	134	81	162
Cr (VI) (mg/kgTS)	<0.5	<0.5	<0.5	<0.5	n.d.	n.d.	n.d.
Hg (mg/kgTS)	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4
Ni (mg/kgTS)	16	12	17	13	21	13	21
Pb (mg/kgTS)	7.7	6.9	<5	7.4	8.1	5.1	9.9
Zn (mg/kgTS)	738	780	512	891	816	432	892

Germination index

The germination index, using lettuce seeds, was determined following OECD guidelines (OECD, 2006). For this test, aqueous extracts (ratio 1:10, in volume) of all the products were prepared, mixing 100 g of material and 1 L of distilled water at room temperature, and letting stand for 6 hours before filtering. Each extract was diluted 100%, 75%, 50%, 25%, and 0% with distilled water. The pH, conductivity, TAN, and $N\text{-}NO_3^-$ were measured in each extract.

Then, 10 mL of each dilution was introduced into a Petri dish with ten homogeneously distributed lettuce seeds. All the 35 Petri dishes, 5 conditions per product, were on a tray and covered with foil or opaque plastic, and lettuce seeds were maintained at 25ºC for 24 hours. Sprouts were counted and left there for another 48 hours. After 72 hours from the test beginning, the number of germinated seeds and roots' length were registered to calculate the germination index of each material (see supplementary material), compared with blank (growth with distilled water), following Equation 3. A G_i value > 80 % indicates that the product is not toxic for plant growth.

Eq.3 $$G_i = \frac{(mean\ lenght * number\ of\ germinated\ seeds\ from\ extract)}{(mean\ length * number\ of\ germinated\ seeds\ from\ blank)}$$

Plant growth test

A pot experiment (Figure 30) was performed from 5th May to 28th June 2022 using the lettuce variety Maravilla to test the pig manure derived products (DD, DAD, DSF, DASF, DM1, DM3, DM6) plus a negative control without adding nitrogen (No-N) and a positive control (Osmocote, a commercial fertilizer). The substrate used in the experiment was a mixture of peat and perlite in a 1:1 ratio. A dose of 150 kgN/ha was applied to all products, trying to keep an stoichiometric relation of N:P:K = 1:0.3:0.2 and thus compensating with K_2O and TSP when needed. The amount of product that was added for each treatment is shown in Table 17.

Table 17. Added amount of fertilizer, based on selected N dose (pot-experiment 1 and 2) cow and pig-derived products. *Wet weight. Abbreviations: CONT, positive control; No-N, negative control without N addition; DD, dried digestate; DAD, dried acidified digestate; DSF, dried solid fraction of digestate; DASF, dried acidified solid fraction of digestate; DM1, mixture 1 (ASF:SLF 3:1); DM3, mixture 3 (ASF:SLF 1:1); DM6, mixture 6 (ASF:SLF 1:8). [1] CONT: Osmocote (commercial fertilizing product)

Product	Amount to add (g/L)*	K_2O added (g/L)	TSP added (45% P)	N:P:K
CONT[1]	0.47	0.06	-	1:0.6:1.5
No-N	x	0.17	0.03	0:0.25:1.5
DD	1.69	0.12	-	1:0.7:1.5
DAD	1.18	0.15	-	1:0.3:1.5
DSF	2.45	0.15	-	1:1.1:1.5
DASF	2.78	0.14		1:1.0:1.5
DM1	2.54	0.13	-	1:1.9:1.5
DM3	2.76	0.14	-	1:0.8:1.5
DM6	2.23	0.09	-	1:1.6:1.5

Each pot had 1.3L of the mix substrate, 28g (dry weight), and each treatment had $n = 10$. Pots were watered individually via drippers with increasing amounts of water, receiving a total amount of 15L per plant.

Figure 30. Pot experimental setup with *Latuca sativa* (var. Maravilla) in the greenhouse.

Relative chlorophyll content (SPAD) (SPAD-502 Plus, Minolta, Japan) was measured just before harvest. Consecutively, plants were harvested and the diameter of the lettuce, and fresh weight were measured. Plants were dried at 60 °C for 72 hours until constant weight and weighted.

Nutrient recovery of N, P and K from plants was calculated following Equation 4 described in Robles-Aguilar *et al*., (2020):

(Eq.4) $$Nutrient\ recovery = \frac{Nutrient\ uptake\ treatment - Nutrient\ uptake\ NoN}{Nutrient\ applied}$$

Plant, leachate and substrate nutrient content analyses

Dry biomass was ground to particles < 2 mm. The samples were subjected to microwave digestion (CEM MARS 6, USA). The nutrient content and metal concentration in the samples were analyzed by ICP-OES. The TC and TN contents were analyzed using the CN analyzer (Skalar Analytical BV, the Netherlands). The leaching was collected from 3 pots per treatment every 10 days directly from the leachate produced after watering and the evolution of pH, electrical conductivity (EC), NH_4^+, and NO_3^- content was measured.

Statistical analyses

Biomass growth, nutrient recovery, and leachate differences were analyzed by One-way ANOVA analysis with SAS 9.4 (SAS Institute Inc. Cary, USA). Mean separation was done with Tuckey test, a post-hoc test to compare multiple means, to identify the significant differences.

Results

Effect of digested manure derived products on germination

The pH, EC and TAN of all the dilutions are shown in Table 18. The highest levels of TAN were measured for DAD, followed by DM6, DM3 and DM1. Moreover, the highest values of EC were shown for DAD and DM3. Acid pH (4.5) was observed in DAD extracts, DM1 and DM3 (6.1-6.5), while the rest of the treatments presented neutral pH.

Table 18. pH, EC, and TAN of all the extraction concentration of the assay products. Abbreviations: DD, dried digestate; DAD, dried acidified digestate; DSF, dried solid fraction of digestate; DASF, dried acidified solid fraction of digestate; DM1, mixture (ASF:SLF 3:1); DM3, mixture (ASF:SLF 1:1); DM6, mixture (ASF:SLF 1:8).

Treatment	**pH**				**EC (mS/cm)**				**N-NH_4^+ (mg/L)**			
Dilution	25	50	75	100	25	50	75	100	25	50	75	100
DD	7.6	7.8	7.9	8.0	1.5	2.7	4.2	5.5	18	36	56	64
DAD	4.6	4.5	4.5	4.4	5.8	10	14	18	491	980	1,457	1,931
DSF	7.5	7.6	7.7	7.7	0.7	1.2	1.8	2.4	17	34	49	68
DASF	7.0	7.4	7.5	7.5	0.7	1.2	1.7	2.2	11	21	31	54
DM1	6.1	6.1	6.3	6.1	0.7	1.3	1.9	2.4	26	49	90	13
DM3	6.4	6.5	6.5	6.5	2.4	4.5	4.7	9.2	121	125	126	177
DM6	7.3	7.4	7.6	7.5	1.0	1.9	2.7	3.6	100	133	137	65

The germination index was calculated using Eq. 2. All the derived pig manure products had a G_i > 80% for all the dilutions, except for DAD and DD-100, which had less than 50% germination. Regarding the mixtures, DM1 showed G_i > 80%; however, Gi for DM3 and DM6 between 60-80%. The highest G_i values were obtained at 50% dilution for DD, DSF and DASF (Figure 31).

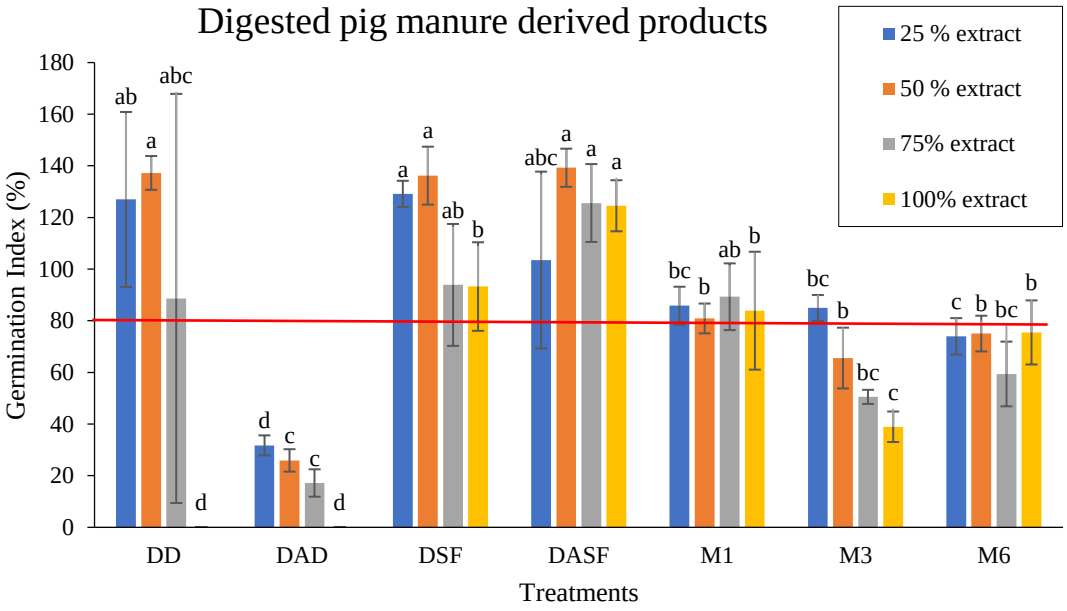


Figure 31. Germination index (%) of pig manure derived fertilizers. Abbreviations: DD, dried digestate; DAD, dried acidified digestate; DSF, dried solid fraction of digestate; DASF, dried acidified solid fraction of digestate; DM1, mixture 1 (ASF:SLF 3:1; acidified solid fraction : low nitrogen stream from the stripping unit); DM3, mixture 3 (ASF:SLF 1:1); DM6, mixture 6 (ASF:SLF 1:8).

Plant and soil analyses

The plant-available nutrients, EC, and pH measured in the growing medium after mixing the respective fertilizers are presented in Table 19.

After applying the pig manure-derived products, the TN concentration in the substrate varied between 0.6% and 0.7% in all products, while TC concentration varied between 32% and 39%. Furthermore, these products led to a pH < 6 (5.2-6.0) in the growing medium, representing a decrease in the preferred substrate pH for lettuce growth, between 6.0-7.0 (Santos *et al*., 2022) and an EC < 1 mS/cm (0.4-0.8 mS/cm), lower than the ideal value for hydroponic growth (1.8 mS/cm) (Teodor *et al*., 2021).

Furthermore, heavy metals from the Spanish legislation (Real Decreto 1310/1990), evaluated as potential toxic elements (PTE) in the Council Directive 86/278/EEC in soils were determined (Cd, Cu, Ni, Pb, Zn, Cr). All concentrations of the mentioned PTE were below the legislation limits of both regulations.

Table 19. pH, EC, nutrients, heavy metals. and potential toxic elements (PTE) measured in the growing medium after the addition of the fertilizing products, expressed as dry matter content. Abbreviations: No-N, control without N; CONTROL, Osmocote; DD, dried digestate; DAD, dried acidified digestate; DSF, dried solid fraction of digestate; DASF, dried acidified solid fraction of digestate; M1, mixture 1 (ASF:SLF 3:1); M3, mixture 3 (ASF:SLF 1:1); M6, mixture 6 (ASF:SLF 1:8).

Pre-cultivation	**CONTROL**	**No-N**	**DD**	**DAD**	**DSF**	**DASF**	**DM1**	**DM3**	**DM6**
pH	5.4	5.8	6.0	5.5	5.6	5.6	5.5	5.2	5.4
EC (mS/cm)	0.6	0.5	0.4	0.7	0.5	0.4	0.4	0.8	0.4
TN (%)	0.7	0.6	0.7	0.7	0.6	0.6	0.6	0.7	0.7
TC (%)	32	34	36	39	34	34	33	38	35
N-NO3- (mg/kg)	441	37	20	96	51	88	102	75	53
Ca (g/kg)	15	15	14	16	18	12	22	19	11
K (g/kg)	0.7	1.5	1.2	1.4	1.6	1.5	1.2	1.1	2.2
Mg (g/kg)	0.8	0.7	0.7	0.7	0.8	0.6	3.8	3.2	0.6
Na (g/kg)	2.0	2.7	3.3	2.9	4.1	5.5	10	8.0	5.1
P (g/kg)	0.2	0.3	0.3	0.5	1.7	0.4	0.3	0.2	0.4
S (g/kg)	1.1	1.2	1.1	2.3	1.4	1.1	5.7	3.6	1.4
Al (mg/kg)	705	809	872	952	1782	933	1112	1206	974
Cd (mg/kg)	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1
Cr (mg/kg)	3.6	4.1	5.1	6.6	9.0	6.8	4.4	5.0	5.4
Cu (mg/kg)	8.5	1.8	2.8	2.3	9.0	6.1	8.7	6.7	2.4
Fe (mg/kg)	599	714	858	1582	2698	1103	1171	1047	1184
Mn (mg/kg)	18	16	18	23	39	21	25	23	21
Ni (mg/kg)	2.2	4.4	2.5	5.9	0.7	5.7	3.2	2.9	2.5
Pb (mg/kg)	2.5	n.d.	14	4.2	11	5.6	7.0	0.6	8.5
Zn (mg/kg)	14	11	14	16	61	20	27	24	17

Plant nutrient content and elements with potential toxic concentrations are shown on Table 20, while the plant nutrient recovery for each treatment is presented in Figure 32. Compared to the No-N treatment, all fertilizers increased the recovery of N, K and P, except for DD (K) and DM6

(K and P). The highest recoveries of N were observed in the Control, DSF and DASF fertilizers. Phosphorous recovery was better in DASF and DM3, while potassium recovery was higher in DASF and DSF. On the contrary, DM6 presented the lowest performance in terms of nutrient recovery for all nutrients, followed by DS for K, and DM1 for P.

Table 20. Plant nutrient content concentration of the harvested lettuces and nutrient intake efficiency compared to the No-N fertilizer. Abbreviations: No-N, control without N; DD, dried digestate; DAD, dried acidified digestate; DSF, dried solid fraction of digestate; DASF, dried acidified solid fraction of digestate; M1, mixture 1 (ASF:SLF 3:1); M3, mixture 3 (ASF:SLF 1:1); M6, mixture 6 (ASF:SLF 1:8); No-N, substrate without any addition of nitrogen; n.d., non-detected. All values expressed per dry matter content.

Treatment	**No-N**	**Control**	**DD**	**DAD**	**DSF**	**DASF**	**M1**	**M3**	**M6**
Element	% dm								
TN	0.6	0.7	0.7	0.8	0.7	0.6	0.8	0.8	0.7
TC	30	32	32	32	30	30	37	36	36
Nutrient recovery									
TN	-	3.4	0.4	1.0	1.9	2.2	1.5	1.5	0.3
TP	-	8.4	3.4	4.8	2.0	14	0.4	14	-
TK	-	7.5	-	4.2	13	24	4.1	1.7	-
	g/kg dm								
Ca	14	14	14	16	15	14	13	12	20
K	17	11	15	16	18	18	16	15	14
Mg	3.3	3.8	3.5	4.0	3.6	3.4	3.1	3.8	3.4
Na	9.4	12	9.9	11	9.4	8.7	8.6	10	8.9
P	1.0	1.0	1.1	1.5	1.6	1.9	0.9	1.6	0.7
S	1.8	1.8	1.7	2.0	1.7	1.4	1.8	1.8	2.0
	mg/kg dm								
Al	115	160	167	214	136	118	122	238	162
Cd	0.3	0.3	0.3	0.3	0.3	0.1	0.4	0.3	0.5
Cr	1.0	1.1	0.9	1.0	0.7	0.8	0.6	1.0	0.9
Cu	8.9	8.2	9.3	10	7.2	12	8.3	11	13
Fe	102	152	151	188	121	206	110	212	140
Mn	101	102	109	111	102	88	120	108	126
Ni	1.1	0.4	0.7	1.2	0.7	0.9	1.2	1.2	0.9
Pb	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.4
Zn	32	41	44	49	37	32	50	52	54

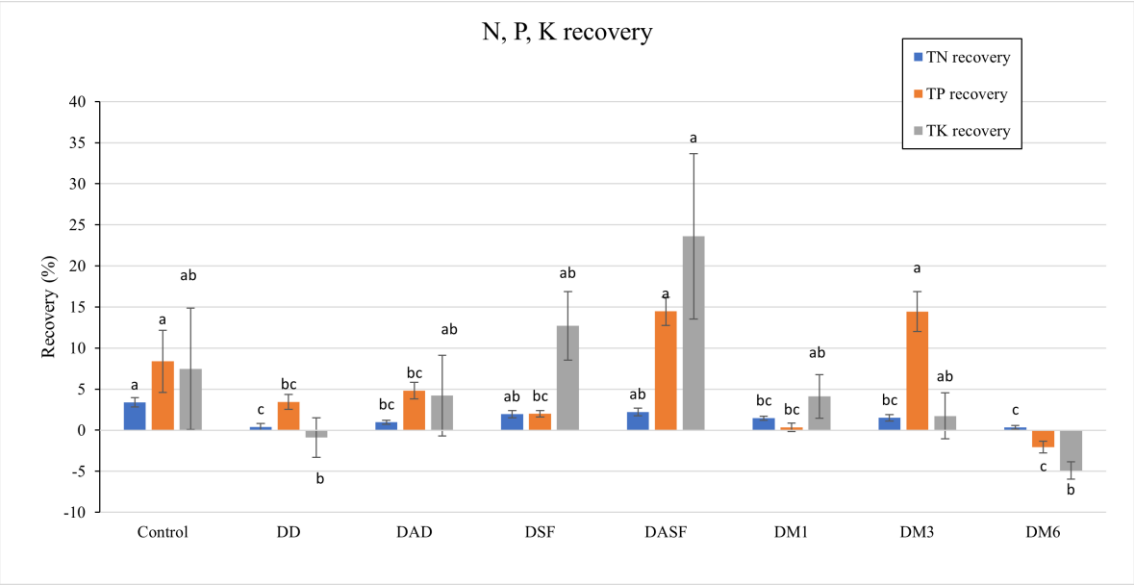


Figure 32. Plant nutrient recovery (N, K, P) for each treatment compared to No-N. Abbreviations: No-N, control without N; DD, dried digestate; DAD, dried acidified digestate; DSF, dried solid fraction of digestate; DASF, dried acidified solid fraction of digestate; DM1, mixture 1 (ASF:SLF 3:1); DM3, mixture 3 (ASF:SLF 1:1); DM6, mixture 6 (ASF:SLF 1:8); No-N, substrate without any addition of nitrogen. Within nutrients, bars with different letters are statistically different.

Effect of biobased fertilizers on plant performance

The effect of the fertilizers on the lettuce growth was determined by analyzing fresh weight, dry weight, diameter, and relative leaf chlorophyll content (SPAD) of the lettuces after 8 weeks (Table 25). DAD and DM3 did not significantly differ from the positive control regarding fresh weight (46 g Control, 43 g DAD, 41g DM3). Moreover, DAD was also not significantly different from the control in terms of dry weight, 6.4 g (DAD) compared to 7.0 g (Control), while the rest of the products presented lower values than the control. The mixtures DM1, DM3, and DM6 presented higher SPAD than the rest of the organic fertilizers, with no significant differences compared to the Control (Table 21).

Table 21. Fresh weight, dry weight, diameter and SPAD of lettuces after 8 weeks growth with the different fertilizer treatments. Abbreviations: DD, dried digestate; DAD, dried acidified digestate; DSF, dried solid fraction of digestate; DASF, dried acidified solid fraction of digestate; DM1, mixture 1 (ASF:SLF 3:1); DM3, mixture 3 (ASF:SLF 1:1); DM6, mixture 6 (ASF:SLF 1:8); No-N, substrate without any addition of nitrogen. Different letters are statistically different.

	Fresh weight (g)	Dry weight (g)	Diameter (cm)	SPAD
(Positive) Control	46 ± 3.3^{a}	7.0 ± 0.5^{a}	17 ± 0.3^{a}	25 ± 1.5^{a}
DD	35 ± 1.5^{cd}	5.0 ± 0.2^{cd}	15 ± 0.3^{b}	21 ± 0.9^{abcd}
DAD	44 ± 1.7^{ab}	6.4 ± 0.2^{ab}	16 ± 0.2^{ab}	16 ± 0.9^{cd}
DSF	37 ± 0.4^{cd}	5.3 ± 0.1^{cd}	16 ± 0.2^{ab}	17 ± 0.8^{bcd}
DASF	38 ± 1.2^{bcd}	5.7 ± 0.2^{bc}	16 ± 0.1^{ab}	16 ± 1.3^{d}
DM1	33 ± 0.8^{cde}	5.3 ± 0.2^{cd}	16 ± 0.3^{ab}	24 ± 1.6^{ab}
DM3	41 ± 1.6^{abc}	5.7 ± 0.2^{bc}	15 ± 0.3^{b}	22 ± 2.1^{abc}
DM6	27 ± 1.9^{e}	4.6 ± 0.2^{d}	16 ± 0.2^{ab}	22 ± 1.7^{abcd}
No-N	31 ± 0.9^{de}	4.5 ± 0.1^{d}	15 ± 0.2^{b}	19 ± 1.7^{abcd}

Contrary to the weight, plant diameter was reduced significantly compared to the positive control at the DD and DM3 treatments (17cm, 15cm, and 15cm, respectively). Concerning SPAD values associated with chlorophyll content and thus N uptake, DD, DM1, DM3 and DM6 presented the highest SPAD values, while DAD, DSF and DASF were the lowest.

Effect of biobased fertilizers on leachate

The total NO_3^- and $N\text{-}NH_4^+$ content in the leachate (Figure 33) varied depending on the fertilizer used between 7 – 32 mg of $N\text{-}NH_4^+$ and 16 – 24 mg of NO_3^-, with the higher $N\text{-}NH_4^+$ content observed in the Control treatment and the higher NO_3^- content in the DD. On the other hand, DM3 and DAD leachate presented the lowest content of NO_3^- and DM3 of $N\text{-}NH_4^+$. Even though there are no significant differences ($p > 0.05$), there are important differences in terms of $N\text{-}NH_4^+$ content between the biobased fertilizers and the control (except for DM6). The statistical analysis of NO_3^- content in the leaching showed significant differences ($p < 0.05$) between No-N and DD compared to DSF, DAD, and DM3.

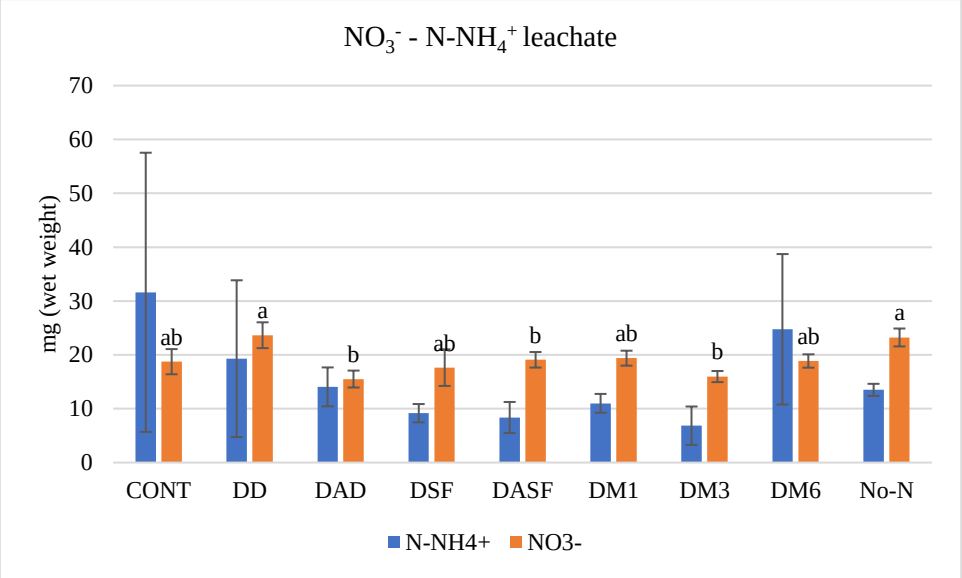


Figure 33. Total amount of $N\text{-}NH_4^+$ and NO_3^- leachate for each fertilizing treatment. Abbreviations: DD, dried digestate; DAD, dried acidified digestate; DSF, dried solid fraction of digestate; DASF, dried acidified solid fraction of digestate; M1, mixture 1 (ASF:SLF 3:1); M3, mixture 3 (ASF:SLF 1:1); M6, mixture 6 (ASF:SLF 1:8); No-N, substrate without any addition of nitrogen. Within nutrients, bars with different letters are statistically different.

Discussion

Germination and phytotoxicity evaluation

In general, all the derived pig manure products had a G_i > 80% for all the dilutions, except for DAD and 100% DD which had less than 50% germination. According to phytotoxicity limits, 80% germination and above is considered non-toxic, and less than 50% G_i is regarded as highly toxic and unsuitable for agricultural purposes (Barral and Paradelo, 2011; Luo *et al*., 2018). Regarding the mixtures, DM1 (ASF: SLF = 3:1) did not show toxicity (G_i > 80%); however, DM3 and DM6, due to their increased concentration of the stripped liquid fraction (ASF:SLF: 1:1 and 1:8, respectively) showed medium toxicity to the lettuce seeds. The toxicity could be related to the high levels of EC present in the extracts, between 5.5 mS/cm for DD100% to 18 mS/cm for DAD. Similar results have been reported in the literature, with Alburquerque *et al*. (2012b) finding that germination of cress and lettuce seeds was inversely correlated with EC.

According to Ünlükara *et al*. (2008), lettuce appeared to have a threshold limit of 1.1 mS/cm. After that point, the relative yield started to decrease. However, in this study, concentrations up to 2.2-2.7 mS/cm represented a G_i higher than 100%, for example, in the case of DASF or DD with 100% and 50% extraction, respectively. At higher EC concentrations, the germination index decreases, and toxicity appears from 4.7 mS/cm. Poor germination indexes were found by Anjum *et al*. (2018) for fresh digestate in wheat seeds related to an EC of 3.6 mS/cm. Their products reached a G_i lower than 15% with the 5% untreated digestate extraction. After an aerobic post-treatment, G_i increased to 45%, a value which still denotes toxicity (G_i < 70%). Da Ros *et al*.

(2018) obtained G_i > 70% with L. *sativum* until 15% digestate extraction, 25% for S. alba and 10% with S. *saccharatum* related to the presence of NH_4^+ and EC as inhibitors. The used digestate had an EC of 5.75 mS/cm, a higher value than most of the products in this study. Coelho *et al*. (2018) determined that the seed germination with L. *sativum* was suppressed with concentrations of (liquid) digestates > 50%, mainly related to EC, with similar results to the ones found in this study with DD.

Another factor that might have influenced seed germination is the TAN concentration. That could have been the case of DAD, which besides having the highest value of EC (18 mS/cm), also had 10 times higher TAN concentration than the rest of the products (Table 16).

Moreover, DD, DSF-25, DSF-50, and DASF stimulated the germination, G_i > 120%, as defined by Da Ros *et al*. (2018). Stimulation observed when applying DASF could be related to the high concentration of $N\text{-}NH_4^+$ (10 times higher than DD and DSF) due to the acidification (Moure-Abelenda *et al*., 2022) without having an excessive EC.

Evaluation of the pot trials and efficiency of the organic fertilizers

Compared to the No-N (negative control) treatment, except for DM6, all the fertilisers had a positive effect on lettuce weight (both wet and dry). Nicoletto *et al*. (2014) used digestate to grow different varieties of lettuce and indicated that the growth compared to the control was different depending on the variety. For butterhead lettuce, growth with digestate was 37% less than the control, and for batavia lettuce, growth with digestate was 5% lower than the control, similar values to the ones presented in this work (4% DAD and 20% for DD). These differences in yield could be correlated to the different timing of nitrogen release and its availability (mineral nitrogen) (Mengel, 1996), as in control TAN was higher than the rest of fertilizers (8.2% of the fertilizer compared to 3.5% in the best scenario with the biobased fertilisers). Montemurro (2010) used two digestates from a wine distillery to grow *Lactuca sativa* (var. *longifolia*), increasing their yield compared to the non-fertilised control by 12 - 19%, similar to this work. When digestate was applied to a hydroponic culture of lettuce, the edible part yield was considerably low compared to the synthetic fertilizer, 78%. However, it increased the total content of polyphenols, antioxidants and soluble sugars (Faran *et al*., 2023). As discussed for the germination results, the application of the biobased fertilizer as an extract allows all the salts to be released at once difficult plant germination and growth. Using it for hydroponics appeared counterproductive, but when using the fertilizer in soil, this can open a door to investigate the benefits of digestate and fertilizer derived.

The heavy metals and PTE were compared to the available legislation. Compared to Real Decreto 1310/1990 and the Council Directive 86/278/EEC, which establishes the limits of heavy metals in soils, none of the regulated parameters were exceeded. Regarding the Commission Regulation

(EU) No 420/2011 of 29th April 2011 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs, limits for Pb and Cd were established at 0.3 and 0.2 mg/kg of fresh weight. None of them was surpassed by the produced lettuces in this study. Generally, leafy vegetables, like lettuce, accumulate more potential toxic elements than fruity ones (Rossini-Oliva and López-Núñez, 2021). However, no existing regulation or health-based standards estimate the risk of their accumulation.

Plant nutrient uptake

In this study, most products derived from pig manure digestate increased the recovery of NPK nutrients, in line with similar biobased or organic fertilizers that have proven a positive effect and produce crops fitting the required standards. (Cen *et al*., 2020; Robles-Aguilar *et al*., 2020; Wu *et al*., 2020). There were some deficiencies in nutrient uptake in the DD treatment (for potassium) and DM6 (for potassium and phosphorus), probably related to the EC, as high EC in soil can create osmotic stress, reducing plant water and nutrient uptake, limiting the availability of nutrients to plant roots (Ding *et al*., 2018).

The highest recoveries of N were observed in DSF and DASF. This was unexpected, as the most available nitrogen is in the liquid fraction (Czekala, 2022). On the other hand, the lowest N recovery was determined in DD, DAD and M6, and all of them presented a low value of Cu in the substrate, which has been proven to increase the uptake of N significantly (Cui *et al*., 2022).

Phosphorus recovery was higher in DASF and DM3, while we were expecting less phosphorous in the plants fertilized with solid fraction derivate products, as liquid fraction contains more available phosphorus, 71% of P_2O_5 of TP, according to Tambone *et al*. (2017). Acidification of the products could play an important role in phosphorous availability, as it increases with acidic pHs (Cerozi and Fitzsimmons, 2016). Nevertheless, even when the pH is acid, and the predominant form of phosphorus is orthophosphate, the presence of Al and Fe produces insoluble compounds (Espinosa and Molina, 2015), which might have happened with the substrates with DAD and DSF.

The K recovery at the DASF and DSF treatments was higher than at the DAD and DD, while most of the K should be distributed after centrifugation to the liquid fraction (Möller and Müller, 2012; Tambone *et al*., 2017). However, the OM content is higher in solid fraction (Czekala, 2022), contributing to potassium availability (Bader *et al*., 2021; Taiwo *et al*., 2018).

Leaching performance

The concentration of $N\text{-}NH_4^+$ in the leachate of the different fertilizers did not significantly vary compared to both controls. Goberna *et al*. (2011) indicate that using digestate as fertilizer did not increase leaching losses. Moreover, a decreasing $N\text{-}NH_4^+$ trend was found for all the products

compared to the Positive Control, regarding NO_3^-, DASF, DAD, and DM3 presented lower losses than the rest of the products. These products were acidified during their production, enhancing ammonium form present on the fertilizers, less susceptible to leach, that will be converted to nitrate slower than in the non-acidified products (Yuliusman *et al*., 2018).

In addition, the products of this study were introduced to the substrate in a dry form, and thus, the nutrients were released with their dilution while watering the plants, acting as a slow-release fertilizer, reducing the possibility of high leachates (Rathnappriya *et al*., 2022).

Conclusions

This work evaluated the performance of different biobased fertilizers derived from pig manure digestate dried with greenhouse solar drying technology on lettuce growth.

After the germination assay, only DAD and 100% concentration of DD and DM3 presented phytotoxicity, mainly due to high levels of EC, as the salts directly affect the seed germination instead of being released slowly in the form of dried fertilizers. The rest of the fertilizers did not produce any toxicity effect on germination, with DASF showing a biostimulation effect (G_i > 120%).

When the products were applied in the solid form in the pot trial, the best performance of the edible part was related to DAD, DM3, and DASF. DSF, DASF and DM3 presented the highest nutrient recovery, always under the performance of the control, as these fertilizers can be described as slow-release.

Regarding leachate, all the fertilizers had similar behaviour to the No-N treatment and fewer N-NH_4^+ losses (trend) than the control. However, regarding NO_3^- losses, DASF, DAD and DM3 presented the lowest values.

Moreover, all the fertilizers fit the current regulations of heavy metals and PTE in soil and plants' edible parts.

Therefore, it can be concluded that the tested biobased fertilizers proved to be suitable for lettuce production, with DASF the one with better performance. More studies with different crops and field applications should be done to determine their performance in broad scenarios.

References

Alburquerque, J.A., de la Fuente, C., Campoy, M., Carrasco, L., Nájera, I., Baixauli, C., Caravaca, F., Roldán, A., Cegarra, J., Bernal, M.P. 2012. Agricultural use of digestate for horticultural crop production and improvement of soil properties, European Journal of Agronomy, Volume 43, Pages 119-128, ISSN 1161-0301, https://doi.org/10.1016/j.eja.2012.06.001.

Alburquerque, J.A., de la Fuente, C., Ferrer-Costa, A., Carrasco, L., Cegarra, J., Abad, M., Bernal, M.P. 2012. Assessment of the fertiliser potential of digestates from farm and agroindustrial residues, Biomass and Bioenergy, Volume 40, Pages 181-189, ISSN 0961-9534, https://doi.org/10.1016/j.biombioe.2012.02.018.

Anjum, M., Qadeer, S., Khalid, A. 2018. Anaerobic Co-digestion of Catering and Agro-Industrial Waste: A Step Forward Toward Waste Biorefinery. Front. Energy Res. 6:116. doi:10.3389/fenrg.2018.00116.

Anuari de dades meteorològiques 2020. Servei Meteorològic de Catalunya. https://www.meteo.cat/wpweb/climatologia/serveis-i-dades-climatiques/series-climatiques-historiques/. Accessed in April 2021.

Bader, B.R., Taban, K. Fahmi, A.H., Abood, M.A., Hamdi, G.J. 2021. Potassium availability in soil amended with organic matter and phosphorous fertiliser under water stress during maise (Zea mays L) growth, Journal of the Saudi Society of Agricultural Sciences, Volume 20, Issue 6, 2021, Pages 390-394, ISSN 1658-077X, https://doi.org/10.1016/j.jssas.2021.04.006.

Barral, M.T., Paradelo, R. 2011. A review on the use of phytotoxicity as a compost quality indicator. Dyn Soil Dyn Plant. 2011;5:36–44.

Buckwell, A. Nadeu, E. 2016. Nutrient Recovery and Reuse (NRR) in European agriculture. A review of the issues, opportunities, and actions. RISE Foundation, Brussels.

Cen Y, Guo L, Liu M, Gu X, Li C, Jiang G. 2020. Using organic fertilisers to increase crop yield, economic growth, and soil quality in a temperate farmland. PeerJ. 2020 Aug 19;8: e9668. doi: 10.7717/peerj.9668.

Cerozi, B.d S., Fitzsimmons, K. 2016.The effect of pH on phosphorus availability and speciation in an aquaponics nutrient solution, Bioresource Technology,Volume 219, 2016, Pages 778-781, ISSN 0960-8524, https://doi.org/10.1016/j.biortech.2016.08.079.

Coelho J.J., Prieto, M.L., Dowling, S., Hennessy, A., Casey, I., Woodcock, T., Kennedy, N. 2018. Physical-chemical traits, phytotoxicity and pathogen detection in liquid anaerobic digestates. Waste Manag. Aug; 78:8-15. doi: 10.1016/j.wasman.2018.05.017.

COMMISSION REGULATION (EU) No 420/2011 of 29th April 2011 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs.

Council Directive 86/278/EEC of 12th June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture.

Cui, X., He, H., Hu, S., Zhang, B., Cai, H. 2022. Synergistic Interaction between Copper and Nitrogen-Uptake, Translocation, and Distribution in Rice Plant. Plants 2022, 11, 2612. https://doi.org/ 10.3390/plants11192612.

Czekała, W. 2022. Digestate as a Source of Nutrients: Nitrogen and Its Fractions. Water 2022, 14, 4067. https://doi.org/10.3390/w14244067.

Da Ros, C., Libralato, G., Ghirardini, A.V., Radaelli, M., Cavinato, C. 2018. Assessing the potential phytotoxicity of digestate from winery wastes. Ecotoxicol Environ Saf. 2018 15th April; 150:26-33. doi: 10.1016/j.ecoenv.2017.12.029.

Ding, X., Jiang, Y., Zhao, H., Guo, D., He, L., Liu, F., Zhou, Q., Nandwani, D., Hui, D., Yu, J. 2018. Electrical conductivity of nutrient solution influenced photosynthesis, quality, and antioxidant enzyme activity of pakchoi (Brassica campestris L. ssp. Chinensis) in a hydroponic system. PLoS One. 2018 Aug 29;13(8):e0202090. doi: 10.1371/journal.pone.0202090.

Espinosa, J., Molina, E. 2015. Acidez y encalado de los suelos (Soil acidity and liming). Doi: 10.13140/2.1.3888.9281.

Faran, M., Nadeem, M., Manful, C.F., Galagedara, L., Thomas, R.H., Cheema, M. 2023. Agronomic Performance and Phytochemical Profile of Lettuce Grown in Anaerobic Dairy Digestate. Agronomy, 13, 182. https://doi.org/10.3390/agronomy13010182.

Goberna, M., Podmirseg, S.M., Waldhuber, S., Knapp, B.A., García, C., Insam, H. 2011. Pathogenic bacteria and mineral N in soils following the land spreading of biogas digestates and fresh manure, Applied Soil Ecology, Volume 49, Pages 18-25, ISSN 0929-1393, https://doi.org/10.1016/j.apsoil.2011.07.007.

Köninger, J., Emanuele, L., Panagos, P., Mrinalini, K., Orgiazzi, A., Briones, M. 2021. Manure management and soil biodiversity: Towards more sustainable food systems in the EU. Agricultural Systems. 194. 103251. 10.1016/j.agsy.2021.103251.

Lencioni, G., Imperiale, D., Cavirani, N, Marmiroli, N., Marmiroli, M. 2016. Environmental application and phytotoxicity of anaerobic digestate from pig farming by in vitro and in vivo trials. Int. J. Environ. Sci. Technol. 13, 2549–2560. https://doi.org/10.1007/s13762-016-1088-y.

Luo, H., Dewitte, K., Landschoot, S., Sigurnjak, I., Robles Aguilar, A., Michels, E., De Neve, S., Haesaert, G., Meers, E. 2022. Benefits of biobased fertilisers as substitutes for synthetic nitrogen fertilisers: Field assessment combining minirhizotron and UAV-based spectrum sensing technologies. Frontiers in Environmental Science. 10. 988932. 10.3389/fenvs.2022.988932.

Luo, Y., Liang, J., Zeng, G.M., Chen, M., Mo, D., Li, G.X., Zhang, D.F. 2018. Seed germination test for toxicity evaluation of compost: its roles, problems and prospects. Waste Manag. 2018; 71:109–114. doi: 10.1016/j.wasman.2017.09.023.

Mengel, K. 1996. Turnover of organic nitrogen in soils and its availability to crops. Plant Soil 181, 83–93. https://doi.org/10.1007/BF00011295.

Möller, K., Müller, T. 2012. Effects of AD on Digestate Nutrient Availability and Crop Growth: A Review. Eng. Life Sci. 2012, 12, 242–257.

Montemurro, F. 2010. Are organic N fertilising strategies able to improve lettuce yield, use of nitrogen and N status? Journal of Plant Nutrition, 33:13, 1980-1997, Doi: 10.1080/01904167.2010.512056.

Moure-Abelenda, A., Semple, K.T., Herbert, B.M.J., Aggidis, G., Aiouache, F. 2022. Valorisation of agrowaste digestate via addition of wood ash, acidification, and nitrification. Environ. Technol. Innov. Article 102632, 10.1016/j.eti.2022.102632.

Nicoletto, C., Santagata, S., Zanin, G., Sambo, P. 2014. Effect of the AD residues use on lettuce yield and quality, Scientia Horticulturae, Volume 180, Pages 207-213, ISSN 0304-4238, https://doi.org/10.1016/j.scienta.2014.10.028.

OECD. 2006. Guideline for the Testing of Chemicals No. 208. Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test. Paris: Organization for Economic Cooperation and Development.

OECD. 2021. Seed Schemes. Rules and Regulations. Schemes for the Varietal Certification or the Control of Seed Moving in International Trade.

Rathnappriya, R.H.K., Sakai, K., Okamoto, K., Kimura, S., Haraguchi, T., Nakandakari, T., Setouchi, H., Bandara, W.B.M.A.C. 2022. Examination of the Effectiveness of Controlled Release Fertilizer to Balance Sugarcane Yield and Reduce Nitrate Leaching to Groundwater. Agronomy 2022, 12, 695. https://doi.org/10.3390/agronomy12030695.

Real Decreto 1310/1990, de 29 de octubre, por el que se regula la utilización de los lodos de depuración en el sector agrario. https://www.boe.es/eli/es/rd/1990/10/29/1310.

Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5th June 2019 laying down rules on the making available on the market of EU fertilising products and

amending Regulations (EC.) No 1069/2009 and (EC.) No 1107/2009 and repealing Regulation (EC.) No 2003/2003.

Riva, C., Orzi, V., Carozzi, M., Acutis, M., Boccasile, G., Lonati, S., Tambone, F., D'Imporzano, G., Adani, F. 2016. Short-term experiments in using digestate products as substitutes for mineral (N) fertiliser: Agronomic performance, odours, and ammonia emission impacts, Science of The Total Environment, 547, pp. 206–214. doi: https://doi.org/10.1016/j.scitotenv.2015.12.156.

Robles-Aguilar, A.A, Grunert, O., Hernandez-Sanabria, E., Mysara, M., Meers, E., Boon, N., Jablonowski, N.D. 2020 Effect of Applying Struvite and Organic N as Recovered Fertilisers on the Rhizosphere Dynamics and Cultivation of Lupine (Lupinus angustifolius). Front. Plant Sci. 11:572741. doi: 10.3389/fpls.2020.572741.

Santos, A.F., Veríssimo, A.M., Brites, P., Baptista, F.M., Góis, J.C., Quina, M.J. 2022. Greenhouse Assays with Lactuca *sativa* for Testing Sewage Sludge-Based Soil Amendments. Agronomy 2022, 12, 209. https://doi.org/10.3390/agronomy12010209.

Taiwo, A.A., Adetunji, M.T., Azeez, J.O., Elemo, K.O. 2018. Kinetics of potassium release and fixation in some soils of Ogun State, Southwestern, Nigeria as influenced by organic manure. Int J Recycl Org Waste Agricult 7, 251–259. https://doi.org/10.1007/s40093-018-0211-0.

Tambone, F., Orzi, V., D'Imporzano, G., Adani, F. 2017. Solid and liquid fractionation of digestate: Mass balance, chemical characterisation, and agronomic and environmental value, Bioresource Technology, Volume 243, 2017, Pages 1251-1256, ISSN 0960-8524, https://doi.org/10.1016/j.biortech.2017.07.130.

Teodor, R., Moraru, P. Mintas, O. 2021. Influence of environmental and nutritional factors on the development of lettuce (Lactuca sativa L.) microgreens grown in a hydroponic system: A review. Notulae Botanicae Horti Agrobotanici Cluj-Napoca. 49. 12427. 10.15835/nbha49312427.

Ünlükara, A., Cemek, B., Karaman, S., Erşahin, S. 2008. Responseof lettuce (Lactuca sativa var. crispa) to salinity of irrigation water, New Zealand Journal of Crop and Horticultural Science, 36:4, 265-273, DOI: 10.1080/01140670809510243.

Vergote, T.L.I., Bodé, S., De Dobbelaere, A.E.J., Buysse, J., Meers, E., Volcke, E.I.P. 2020. Monitoring methane and nitrous oxide emissions from digestate storage following manure mono-digestion, Biosystems Engineering, Volume 196, Pages 159-171, ISSN 1537-5110, https://doi.org/10.1016/j.biosystemseng.2020.05.011.

Walsh, J.J., Jones, D.L., Edwards-Jones, G., Williams, A.P. 2012. Replacing inorganic fertiliser with anaerobic digestate may maintain agricultural productivity at less environmental cost J. Plant Nutr. Soil Sci., 175 (2012), pp. 840-845. https://doi.org/10.1016/j.wasman.2022.10.025.

Wu, L., Jiang, Y., Zhao, F., He, X., Liu, H. 2020. Increased organic fertiliser application and reduced chemical fertiliser application affect the soil properties and bacterial communities of grape rhizosphere soil. Sci Rep 10, 9568. https://doi.org/10.1038/s41598-020-66648-9.

Yuliusman, Amiliana, R.A., Wulandari, P.T., Ramadhan, I.T., Kusumandewi, F.A. 2018. Selection of organic acid leaching reagent for recovery of zinc and manganese from zinc-carbon and alkaline spent batteries IOP Conf. Ser.: Mater. Sci. Eng. 333 012041.

Chapter 9 – Thesis overview, general conclusions and future research perspectives

Thesis overview

There are several strategies and technologies that are suitable to be applied along the agronomic chain in order to reduce emissions and to recover and reuse nutrient from agro-industrial waste streams. However, the individualistic perception of the research in the field of the sustainability of the agri-food system must give way to integrated approaches allowing to introduce the principles of the circular economy and the bioeconomy in the primary sector from farm to fork. This doctoral thesis has contributed to this approach by working at different steps within intensive mixed-farming systems such as modification of animal diets, manure treatment and valorization, and plant production. They were not necessarily linked one after the other, but it can give an idea of how the agricultural chain can be connected and how the studied processes can affect the following step.

Diet modification through precision feeding resulted in a reduction of both N excreted by the animals and a decrease of NH_3 emissions during manure storage, but increased manure CH_4 emissions. A livestock farm treating manure in a biogas plant could take profit from this apparent disadvantage by using a precision feeding system to increase nutrient efficiency and biogas production, while generating a nutrient rich digestate. However, even though enteric CH_4 emission estimation did not vary significantly it is an important parameter to consider, as it is the major source of CH_4 from cattle production. Moreover, although out of the scope of this thesis, manure coming from the conventional and the precision feeding systems were applied to fertilize ryegrass as animal forage, within the same case study of the H2020 project, thus closing the cycle in a mixed farming system (dairy-fodder production).

Since the use of manure or even digestate applied directly to the field produces emissions and nutrient leaching, the application of valorization technologies to improve its fertilizing value and produce more stable products would be required. The alternative implemented in this thesis has been the use of a solar drying system combined with acidification, which has been able to produce bio-based fertilizers from anaerobic digestate with high agronomic value, and a reduction of NH_3 and CH_4 emissions during their production. Afterwards, lettuces has been grown with these fertilizers to test phytotoxicity. The produced plants fit the regulation of heavy metals and PTE both for soils and edible parts of plants.

The use of the produced fertilizers has been tested, again out of the scope of this thesis, by Nascmento *et al*. (2023), applying DAD to canola, pea, barley, triticale and wheat during 3 years of experiment with yield and protein content results comparable to synthetic fertilizers.

General conclusions

At the beginning of this thesis, three specific objectives were presented:

SO1. Improve nutrient use and recovery at animal production level:

Feeding cows under a precision feeding system improves the adjustment of protein and energy requirements, reducing the overconsumption of energy and CP in cows after 80 DIM. Moreover, milk production does not present any difference between both treatments during the studied period of time. All this data suggests that the adjust in crude protein and N content of the feeding directly affect their outputs.

SO2. Reduce GHG and NH_3 emissions from intensive livestock systems:

Manure from cows fed under a precision feeding system tends to reduce by 20% NH_3 and 11% N_2O emissions, and to increase 14% CO_2 and 55% CH_4 respective to cows fed a unique TMR.

Megasphaera elsdenii, and *Prevotella ruminicola* had not been described yet as HAP, just included as deaminating bacteria. However, in this study, their NH_3 production is comparable to the previously described HAP (682 and 348 nMol NH_3/min/mg protein respectively; > 250 - 300 nMol NH_3/min/mg protein). Nevertheless, the bacteriophages isolated from EVAM dairy cows' do not infect the HAP isolated from the same rumina fluid.

The acidification during the drying process of digestate and its solid fraction reduces NH_3 (up to 94%) and CH_4 (up to 57%) emissions, but increases N_2O emissions.

SO3. Increase the fertilizer value of livestock manure and digestate from agro-industrial biogas plants through innovative technological and management strategies:

During the production of dried fertilizers derived from pig manure digestate, DASF and DAD recover more TN and TAN (up to 14 times) than the non-acidified products (DSF and DD).

All products fit with the Regulation EU 2019/1009 as solid organic fertilizers, except for DASF and the mixture DM1 due to higher concentration levels of Zn.

The germination assay shows good results for most of the products except for the ones with higher EC and NH_4^+. In addition, DASF produces a biostimulation effect on lettuce seed germination (Gi > 120%).

In the pot trial, DAD, DM3, and DASF have a performance comparable to the synthetic fertilizer, when referred as the edible part of the plant. DSF, DASF and DM3 show the highest nutrient recovery compared to the rest of products and even similar or higher than the synthetic fertilizer depending on the nutrient.

The fertilizing products have better results than the negative control, showing their potential use as fertilizers. Moreover, all of them fit the regulations of heavy metals and PTE in both soils and edible parts of plants.

Improvement suggestions

Nonetheless, there are some aspects of the thesis that could be improved to produce even a higher impact.

Considering the precision feeding study, the duration time was probably the most critical point of the experiment. On one hand, the dairy cow study lasted 21 days, and it was not enough to evaluate performance parameters, so the lack of impairment of performance for an entire lactation should be demonstrated in animals fed under precision feeding system. On the other hand, nitrous oxide emissions tend to increase after 14 days during storage, as a natural crust is formed in the surface that allows nitrification conditions. Therefore, it would be necessary to evaluate storage during longer periods to determine the effect on N_2O emissions. Furthermore, the increase of CH_4 emissions of precision feeding manure suggested a better methanization potential that should be demonstrated in *in vitro* assays. It becomes necessary to analyze the enteric emissions as well and to try other diet compositions reducing the amount of carbohydrates. Regarding the study of HAP and bacteriophages, the improvement in the isolation and identification of ruminal HAP under anaerobic conditions could be improved, i.e., using anaerobic chambers, to potentially identify more strictly anaerobic microorganisms than the ones described in this work, since rumen is a strict anaerobic environment at constant temperature and pH, or taking samples from cows with different diets to enrich the microbiome range of species. Moreover, to test the prove of concept of the potential use of bacteriophages as a tool to reduce NH_3 emissions, the use of commercial bacteriophages could be a solution to evaluate the reduction or not of NH_3 production in the ruminal system, and to continue the research line following this direction if it is proven using isolated rumen bacteriophages.

One of the critical aspects of the bio-based fertilizers study was that the effect of acidification disappeared during drying, since OM and protons combined are degraded forming other compounds. Therefore, it would be interesting to control acidification through the entire drying process and evaluate the differences in emissions. Although the turning was breaking partially the crust, the homogenization was not perfect, and the material formed layers with different humidity content, which complicated the sampling methodology. This needs to be improved by collecting a better representation of the material in depth, wide and length. Literature shows different growth behaviors of lettuce with digestate depending on the variety. It would be necessary to test the products not only with different lettuce varieties but also with different crops to have a broader understanding of their performance.

Future research perspectives

The following suggestions aim to suggest further research to improve or continue the research line developed in this thesis:

Effect of HAP-bacteriophages in a ruminal simulation system:

In this thesis, the effect of ruminal bacteriophages on HAP bacteria was not proved. However, bacteriophages interactions are widely known (Duan *et al*., 2022; Sahu *et al*., 2022), and bacteriophages affecting *Clostridium sporogenes* have been described in the literature and they are available in bank collections (Mayer *et al*., 2012). The use of more complex systems to evaluate rumen fermentation as RUSITEC could be used to study dynamics on the bacterial community (Wetzels *et al*., 2018) and consequently the effects of anti-biotics, enzymes, or even bacteriophages on them.

Considering the two above mentioned premises, the proposed future research will be focused on the evaluation of known HAP-bacteriophages inoculated in the ruminal simulation system to understand if the NH_3 production and HAP population would decrease, and how it impacts on the microbial community and fermentation, before testing the implementation in *in vivo* assays.

Evaluation of crust microbial population:

In chapter 7, during the drying of digestate derived products, a natural organic crust was formed, which produced changes in material structure and gas emissions. The formation of the crust induces a decrease of water diffusivity (Joardder *et al*., 2016), increasing the time of drying. In addition, crusts allowed anaerobic and aerobic areas, enhancing nitrification and the presence of ammonia oxidizing bacteria (Nielsen *et al*., 2010; Sajeev *et al*., 2018). When the turning machine was modified, crust removal was enhanced and N_2O emissions decreased compared to the previous system.

Kupper *et al*., (2020) observed a significant reduction on NH_3 and a trend of reduction for CH_4 and CO_2 with the formation of a crust, and Hansen *et al*., (2009) analyzed microbial population in dry and wet straw crusts. Both had presence of ammonia oxidizing bacteria, meaning that NH_3 will be reduced and N_2O release will increase, but the dry one also presented CH_4 oxidizing bacteria that could be related to a decrease of CH_4.

As future research in this line, the microbial population of the material surface and natural organic crusts would be evaluated to understand better the mechanisms and conditions leading to the variation of emission during storage and drying so mitigation solutions to modify these populations could be proposed and evaluated.

Acidification effect on microbial population:

The effect of acidification had an impact on NH_3, CH_4, and N_2O emissions, as it has been seen in previous chapters, causing inhibition of methanogens and ammonia oxidizing bacteria. Liu *et al*., (2010), described the inhibition of nitrous oxide reductase when pH was decreased to 5, enhancing the production of N_2O by reducing denitrification. Wang *et al*., (2022) observed that acidification enhanced fungal N_2O production. Nevertheless, the study of dynamic changes on manure/digestate microbial population when acidifying is not well understood (Fangueiro *et al*., 2015; Sigurnjack *et al*., 2017).

Microbial population changes should be studied during manure/digestate storage to understand the dynamics leading to changes in gas emissions.

Crop field trials using bio-based digestate fertilizers:

Soil characteristics have time-space variability, their changes can only be proved after decades, and they are weather/climate dependent. We have data of micro and macronutrients in soil and limit for pollutants, but no optimal values for C and N and their behavior. The effect of crop rotation, management systems, interrelation between soil-hydrosphere-atmosphere, and nutrient use efficiency can only be trust after a long-term field experiment (Körchens, 2006).

The fertilizers produced in the framework of this thesis have been tested in germination and pot trials with positive results. In the framework of Circular Agronomics project, Nascimento *et al*., (2023) applied DAD with favorable results. But still, long-term field application trials would be a good recommendation of future research to be sure about their full performance on crops, but also on soil. However, the rest of the products need to be tested in field experiments.

References

Duan, Y., Young, R., Schnabl, B. 2022. Bacteriophages and their potential for treatment of gastrointestinal diseases. Nat Rev Gastroenterol Hepatol 19, 135–144. https://doi.org/10.1038/s41575-021-00536-z.

Fangueiro, D., Hjorth, M., Gioelli, F. 2015. Acidification of animal slurry–a review. J Environ Manage.149: 46–56. pmid:25463570. DOI: 10.1016/j.jenvman.2014.10.001.

Hansen, R.R., Nielsen, D.A., Schramm, A., Nielsen, L.P., Revsbech, N.P., Hansen, M.N. 2009. Greenhouse gas microbiology in wet and dry straw crust covering pig slurry. J Environ Qual. 2009 Apr 27;38(3):1311-9. doi: 10.2134/jeq2008.0336.

Joardder, U.H., Mohammad, O., Karim, A. Kumar, C., Brown, R. 2015. Porosity: Establishing the Relationship between Drying Parameters and Dried Food Quality. Springer. 10.1007/978-3-319-23045-0.

Kupper, T., Häni, C. , Neftel, A., Kincaid, C., Bühler, M., Amon, B., VanderZaag, A. 2020. NH3 and greenhouse gas emissions from slurry storage - A revie., Agric. Ecosyst. Environ. 300:106963. https://doi.org/10.1016/j.agee.2020.106963.

Liu, B., Mørkved, P.T., Frostegård, Å., Bakken, L.R., 2010. Denitrification gene pools, transcription and kinetics of NO, N2O and N2 production as affected by soil pH. FEMS Microbiol. Ecol. 72, 407–417.

Mayer, M.J., Gasson, M.J., Narbad, A. 2012. Genomic sequence of bacteriophage ATCC 8074-B1 and activity of its endolysin and engineered variants against Clostridium sporogenes. Appl Environ Microbiol. 2012 May;78(10):3685-92. doi: 10.1128/AEM.07884-11.

Nascimento, G. Villegas, D., Cantero-Martínez, C. 2023. Crop diversification and digestate application effect on the productivity and efficiency of irrigated winter crop systems. European Journal of Agronomy. 148. 10.1016/j.eja.2023.126873.

Nielsen, D. A., Nielsen, L. P., Schramm, A., Revsbech, N. P. 2010. Oxygen distribution and potential ammonia oxidation in floating, liquid manure crusts. J. Environ. Qual. 39:1813–1820.

Sajeev, E. P. M., Winiwarter, W., Amon, B. 2018. Greenhouse gas and ammonia emissions from different stages of liquid manure management chains: Abatement options and emission interactions. J. Environ. Qual. 47(1): 30-41.

Sahu, R., Singh, A.K., Kumar, A., Singh, K., Kumar, P. 2022. Bacteriophages concept and applications: A review on phage therapy. Curr Pharm Biotechnol. 2022 Nov 4. doi: 10.2174/1389201024666221104142457.

Sigurnjak, I., Michels, E., Crappé, S., Buysens, S., Biswas, J. Tack, F., De Neve, Ss, Meers, E. 2017. Does acidification increase the nitrogen fertilizer replacement value of bio-based fertilizers? Journal of Plant Nutrition and Soil Science. 180. 10.1002/jpln.201700220.

Wetzels SU, Eger M, Burmester M, Kreienbrock L, Abdulmawjood A, Pinior B, Wagner M, Breves G, Mann E. The application of rumen simulation technique (RUSITEC) for studying dynamics of the bacterial community and metabolome in rumen fluid and the effects of a challenge with Clostridium perfringens. PLoS One. 2018 Feb 7;13(2):e0192256. doi: 10.1371/journal.pone.0192256.