UNIVERSITY OF IOANNINA

UTILIZATION OF RICE CROP RESIDUALS FOR BIOGAS PRODUCTION THROUGH ANAEROBIC DIGESTION AND THE USE OF DIGESTATE AS FERTILIZER FOR ADDED-VALUE CROPS

Kalaitzidis Argyrios

UNIVERSITY OF IOANNINA

SCHOOL OF AGRICULTURE

DEPARTMENT OF AGRICULTURE

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Αξιοποίηση των υπολειμμάτων των ορυζώνων για την παραγωγή βιοαέριου με αναερόβια χώνευση και χρήση του χωνεμένου λύματος στην καλλιέργεια φυτών προστιθέμενης αξίας

by

Argyrios Kalaitzidis

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University of Ioannina, 47100 Arta, Greece

The Examination Committee

Nikolaos E. Korres
Associate Professor
Department of Agriculture
University of Ioannina
(Supervisor)

Panagiotis G. Kougias
Senior Researcher
Soil and Water Resources Institute
Hellenic Agricultural Organization – Dimitra
(Member of the Advisory Committee)

Spyridon Koutroubas
Professor
Department of Agricultural Development
Democritus University of Thrace
(Member of the Advisory Committee)

Theodore E. Matikas
Professor
Department of Chemical and Materials Engineering
University of Ioannina
(Member of the Examination Committee)

Ioannis D. Leonardos
Professor
Department of Biological Applications and Technologies
University of Ioannina
(Member of the Examination Committee)

Ioannis Skoufos
Professor
Department of Agriculture
University of Ioannina
(Member of the Examination Committee)

Sotirios Kalamaras
Assistant professor
Veterinary Department
Aristotle University of Thessaloniki
(Member of the Examination Committee)

To my late father
Nikolaos Kalaitzidis

Η φύσις μηδέν μήτε ατελές ποιεί μήτε μάτην
Αριστοτέλης

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Plagiarism Statement

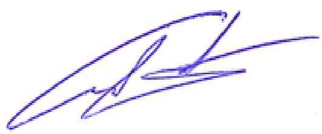
As a Ph.D. student at the University of Ioannina (Greece), specifically in the Department of Agriculture.

I CERTIFY THAT:

The present dissertation records the progress made for the proposed Ph.D. thesis titled *“Utilization of rice crop residuals for biogas production through anaerobic digestion and the use of digestate as fertilizer for added-value crops”*.

This thesis is entirely the product of my own work, and I have not obtained a degree from this university or elsewhere based on this work.

For the record and for any applicable purposes, I sign this document in Arta (Greece), **7th of May 2026.**



Signed: Argyrios Kalaitzidis

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Abstract

Agricultural residues, such as rice straw and livestock slurries, can contribute to climate change and atmospheric pollution if not properly managed. Anaerobic digestion of such residues produces renewable energy in the form of biogas, while mitigating the adverse effects of improper agricultural waste management. However, rice straw and other lignocellulosic biomass are recalcitrant and hence difficult to biodegrade. Therefore, pretreatment prior to digestion has emerged as an effective strategy to enhance their biodegradability. Our study examined the effect of eight thermal and four thermochemical pretreatment methods on the biodegradability of rice straw. Thermal pretreatments involved the use of heat combined with digestate, while thermochemicals involved the use of heat coupled with NaOH. Results showed that thermal pretreatments resulted in minimal increases in methane production, up to 5%, while thermochemical treatments resulted in more substantial increases, up to 15%, compared to the untreated rice straw. Subsequently, co-digestion experiments were performed using the optimum pretreatment methodology with two slurries. Pretreated rice straw was co-digested with cattle slurry and swine slurry separately in different volatile solids (VS) ratios ranging from 50% slurry and 50% pretreated rice straw up to 100% slurry. According to the results of our experiments, co-digestion of pretreated rice straw with cattle slurry resulted in a methane yield increase of up to 14%, while co-digestion with swine slurry increased methane production up to 59%. In both cases, maximum increases in methane yield were achieved when pretreated rice straw was co-digested with each slurry at a 50%:50% VS (w/v) ratio. All experiments were performed at a batch scale. Additionally, we performed a technoeconomic analysis to investigate whether establishing a biogas power plant in Northern Greece would be a profitable endeavor. According to our results, the project is viable, with the net annual profit reaching €1,510,000, with revenue from digestate sales and €955,358 without, comparable to other plants in the Mediterranean region. The payback period, with digestate sales, was estimated at 2.98 years, the return on investment at 33.5% and the profit margin at 62.4% per year, respectively. Likewise, without digestate revenue, the payback period increased to 4.71 years, while the return on investment and profit margin decreased to 21.23% and 51.26% per year, respectively.

In order to examine the robustness of our system against instantaneous and gradual increases in the organic loading rate (OLR), we used three mesophilic lab-scale continuous stirred tank reactors (CSTR). The feedstock used was the optimum co-digestion mixture from our previous batch scale experiments. The first reactor (R1) served as the control and continued to operate throughout the experiment under 2 gVS $L^{-1}d^{-1}$. The OLR in the second reactor (R2) increased gradually from 2 gVS $L^{-1}d^{-1}$ to 3 gVS $L^{-1}d^{-1}$ and then 4 gVS $L^{-1}d^{-1}$, while the OLR in the third reactor (R3) increased instantaneously from 2 gVS $L^{-1}d^{-1}$ to 4 gVS $L^{-1}d^{-1}$ and then 6 gVS $L^{-1}d^{-1}$. According to the results of our experiments, the highest methane yield was achieved at a stable OLR of 2 gVS L^{-1} (353 mL CH_4 gVS^{-1}). Gradual increases resulted in transient process disruptions, showcasing the robustness of our system and its recoverability. Conversely, instantaneous increases up to 6 gVS L^{-1} d^{-1} led to considerable system disruptions, with methane yield decreasing significantly (331 mL CH_4 gVS^{-1}) and CH_4 content in the biogas mixture reducing to 51%. Analysis of total ammonia nitrogen content (TAN) and free ammonia (FA) showed strong inhibition during periods of stress. TAN and FA reached ~3,500 mg L^{-1} and 212 mg L^{-1}, respectively, in R3 when the OLR increased to 6 gVS L^{-1} d^{-1}. Correlation analysis confirmed the strong and negative correlation between methane production and TAN ($r = -0.826$). Notably, chemical oxygen demand (COD) removal efficiency (65–67%) was not significantly hindered by the increases in TAN and OLR. In general, gradual increases proved

far more favorable compared to instantaneous, allowing the system to recover without significant process disruptions.

Digestate is the by-product of anaerobic digestion, rich in macro and micronutrients necessary for plant development. Basil (*Ocimum Basilicum* L.) is one of the most economically important medicinal and aromatic plants (MAPs), widely used in Italian and Greek cuisine. In order to examine the effect of digestate as an alternative fertilizer for basil development, a two-year pot experiment was performed. Our experiment comprised four fertilization regimes: chemical fertilizer (F), digestate (D), combined chemical fertilizer and digestate (1:1, FD), and unfertilized control (C). Several key metrics were estimated, such as plant height, total plant biomass (TB), leaf biomass production (LP), chlorophyll concentration index (CCI), essential oil yield, and composition. Post-harvest soil samples were taken to assess the effect of digestate fertilization on the heavy metal and nutrient content of soil and the concentration of pathogens in the soil and on the leaves of the basil plants. Results showed that FD treatment significantly increased TB (68.2 g plant^{-1}) and LP (52.7 g plant^{-1}), while also demonstrating the highest essential oil production per hectare. Digestate use increased the extractable concentration of crucial macronutrients, phosphorus (P), and potassium (K) by 68.5% and 134.4%, respectively, while also significantly increasing the soil organic matter by 54.7%. The highest essential oil content was observed in the control plants. Importantly, different fertilization regimes influenced secondary metabolite synthesis. In particular, plants treated with digestate exhibited significant increases in sesquiterpenes, especially germacrene D and *β*-elemene. Chemical fertilization increased the synthesis of methyleugenol and *β*-farnesene, both of which are associated with protection from biotic stresses and reproduction. Unfertilized plants showed increased monoterpene synthesis and particularly limonene, eugenol, and linalool, all of which are associated with abiotic stresses, such as cold and drought tolerance, while showing superior antibacterial, antifungal, antioxidant, and antineoplastic activity. Therefore, the absence of fertilizers promoted the formation of stress-alleviating terpenes, mineral fertilizers favored the synthesis of terpenes linked with biotic stresses and reproduction, digestate promoted the synthesis of sesquiterpenes, while FD plants exhibited a more neutral profile. Crucially, *Salmonella* spp. was not detected either in the soil or on the leaves of the plants. *Enterococcus faecalis* and *Escherichia coli* were found in small concentrations in the soil (< 9.1 cfu g^{-1}). In contrast, the concentration of *Enterococcus faecalis* on the leaves was relatively high (91est in D-treated plants and 73 est cfu g^{-1} in FD-treated plants), marginally below the threshold of 100 cfu g^{-1}, for ready-to-eat fruit and vegetables, while concentrations of *E. coli* remained low on the leaves (<9.1 cfu g^{-1}).

Περίληψη

Η μη ορθή διαχείριση των υπολειμμάτων της αγροτικής παραγωγής, όπως τα άχυρα του ρυζιού και τα λύματα της κτηνοτροφίας, δύναται να εντείνουν το φαινόμενο της κλιματικής αλλαγής και της ατμοσφαιρικής ρύπανσης. Η αναερόβια χώνευση αυτών των υπολειμμάτων παράγει ανανεώσιμη ενέργεια ενώ παράλληλα μειώνει τις αρνητικές επιπτώσεις από τη μη ορθή διαχείριση τους. Εν τούτοις, τα άχυρα του ρυζιού και λοιπές λιγνοκυτταρινούχες ενώσεις είναι δυσδιάλυτες και συνεπώς είναι δύσκολο να βιοαποικοδομηθούν. Επομένως, η προεπεξεργασία αυτών των υποστρωμάτων πριν από την χώνευση τους αποτελεί μία επιτυχημένη στρατηγική για την αύξηση της βιοαποικοδομισιμότητας τους. Η παρούσα μελέτη εξέτασε την επίδραση οκτώ θερμικών και τεσσάρων θερμοχημικών μεθόδων στην βιοαποικοδομιση του αχύρου του ρυζιού. Η θερμική προεπεξεργασία πραγματοποιήθηκε με τη χρήση θερμότητας και χωνεμένου λύματος, ενώ η θερμοχημική με τη χρήση θερμότητας παρουσία NaOH. Τα αποτελέσματα έδειξαν ότι οι θερμικές επεξεργασίες οδήγησαν σε χαμηλές αυξήσεις στην παραγωγή μεθανίου, έως 5%, ενώ οι θερμοχημικές επεξεργασίες οδήγησαν σε σημαντικές αυξήσεις έως 15%, σε σύγκριση με το μη-επεξεργασμένο άχυρο ρυζιού. Στη συνέχεια, πραγματοποιήθηκαν πειράματα συγχώνευσης με προεπεξεργασμένο άχυρο ρυζιού (χρησιμοποιώντας την βέλτιστη μεθοδολογία προεπεξεργασίας) και δύο ζωικά λύματα. Κατά την πειραματική διαδικασία προεπεξεργασμένο άχυρο συγχωνεύθηκε με αγελαδινά λύματα και χοιρινά λύματα σε ξεχωριστά πειράματα και σε διάφορες αναλογίες πτητικών στερεών (VS), ξεκινώντας από 50% ζωικά λύματα και 50% προεπεξεργασμένη βιομάζα ρυζιού έως 100% ζωικά λύματα. Σύμφωνα με τα αποτελέσματα της έρευνας μας, η συγχώνευση του προεπεξεργασμένου αχύρου ρυζιού με τα αγελαδινά λύματα οδήγησε σε αύξηση της παραγωγής μεθανίου έως και 14%, ενώ η συγχώνευση με χοιρινά λύματα οδήγησε σε αύξησε της απόδοσης σε μεθάνιο έως και 59%. Οι μέγιστες αυξήσεις στην παραγωγή μεθανίου, ανεξαρτήτου λύματος, προέκυψε όταν η αναλογία προεπεξεργασμένου αχύρου ρυζιού και λυμάτων ήταν 50%:50% VS (w/v). Όλα τα πειράματα ήταν διαλείποντος έργου. Επιπλέον, πραγματοποιήσαμε μία τεχνοοικονομική ανάλυση για να διερευνήσουμε εάν η δημιουργία μιας μονάδας παραγωγής βιοαερίου στη Βόρεια Ελλάδα θα ήταν ένα κερδοφόρο εγχείρημα. Τα αποτελέσματα έδειξαν ότι το εγχείρημα θα ήταν επιτυχημένο με τα μέσα καθαρά ετήσια εισοδήματα να ανέρχονται στο €1,510,000 με τα έσοδα από την πώληση του χωνεμένου λύματος και €955,358 χωρίς, κερδοφορία αντίστοιχη με άλλες μονάδες παραγωγής βιοαερίου στην περιοχή της Μεσογείου. Η περίοδος απόσβεσης με τα κέρδη από την πώληση του χωνεμένου λύματος υπολογίσθηκε σε 2,98 χρόνια, η απόδοση της επένδυσης σε 33,5% και το περιθώριο κέρδους σε 62,4% ανά έτος, αντίστοιχα. Επιπλέον, χωρίς τα έσοδα από την πώληση του χωνεμένου λύματος, η περίοδος απόσβεσης αυξήθηκε σε 4,71 χρόνια, ενώ η απόδοση της επένδυσης και το περιθώριο κέρδους μειώθηκαν σε 21,23% και 51,26% ανά έτος, αντίστοιχα.

Για την διερεύνηση της ανθεκτικότητας του συστήματος μας σε σταδιακές και ακαριαίες αυξήσεις στον ρυθμό οργανικής φόρτισης (OLR) χρησιμοποιήσαμε τρείς εργαστηριακούς μεσόφιλους αντιδραστήρες συνεχούς ανάδευσης (CSTR). Το υλικό τροφοδοσίας αποτελούνταν από το βέλτιστο συνδυασμό προεπεξεργασμένου αχύρου ρυζιού και χοιρινών λυμάτων, σύμφωνα με τα προηγούμενα πειράματα διαλείποντος έργου. Ο πρώτος αντιδραστήρας (R1) αποτελούσε τον «μάρτυρα» του πειράματος και λειτούργησε σε όλη τη διάρκεια του πειράματος με 2 gVS $L^{-1}d^{-1}$. Ο ρυθμός οργανικής φόρτισης στον δεύτερο αντιδραστήρα (R2) αυξήθηκε σταδιακά από 2 gVS $L^{-1}d^{-1}$ σε 3 gVS $L^{-1}d^{-1}$ και τελικά σε 4 gVS $L^{-1}d^{-1}$, ενώ ο OLR στον τρίτο αντιδραστήρα (R3) αυξήθηκε ακαριαία από 2 gVS $L^{-1}d^{-1}$ σε 4 gVS $L^{-1}d^{-1}$ και τελικά σε 6 gVS $L^{-1}d^{-1}$. Σύμφωνα με τα αποτελέσματα της έρευνας μας, η υψηλότερη απόδοση σε μεθάνιο προήλθε όταν ο OLR παρέμεινε στα 2 gVS L^{-1} (353 mL CH_4

gVS^{-1}). Η σταδιακή αύξηση του ρυθμού οργανικής φόρτισης είχε ως αποτέλεσμα παροδικές διαταράξεις στην ομαλή λειτουργία της διαδικασίας, επιδεικνύοντας την ανθεκτικότητα του συστήματος μας και την γρήγορη επαναφορά του σε κατάσταση ισορροπίας. Εν αντιθέσει, οι ακαριαίες αυξήσεις στον OLR έως και 6 gVS L^{-1} d^{-1} είχαν σαν συνέπεια σημαντικές διαταράξεις στην ισορροπία του συστήματος, με την απόδοση σε μεθάνιο να μειώνεται σημαντικά (331 mL CH_4 gVS^{-1}) και το ποσοστό μεθανίου στο μίγμα του βιοαερίου να μειώνεται στο 51%. Ο υπολογισμός των συγκεντρώσεων αμμωνιακού αζώτου (TAN) και ελεύθερης αμμωνίας (FA) έδειξαν ισχυρή παρεμπόδιση της διαδικασίας, με τις συγκεντρώσεις TAN και FA να σταθεροποιούνται στα ~3,500 mg L^{-1} και 212 mg L^{-1}, στον R3 όταν ο OLR αυξήθηκε στα 6 gVS L^{-1} d^{-1}. Η ανάλυση συσχέτισης επιβεβαίωσε την ισχυρή και αρνητική συσχέτιση μεταξύ της παραγωγής μεθανίου και TAN ($r = -0.826$). Αξιοσημείωτο είναι ότι το ποσοστό μείωσης απαιτήσεως οξυγόνου (COD) δεν μειώθηκε σημαντικά (65–67%) παρόλες τις αυξήσεις σε TAN και OLR. Γενικά οι σταδιακές αυξήσεις αποδείχθηκαν πολύ πιο ευνοϊκές σε σύγκριση με τις ακαριαίες, επιτρέποντας το σύστημα να επανακάμψει χωρίς σημαντικά προβλήματα.

Το χωνεμένο λύμα είναι ένα υπό-προϊόν της αναερόβιας χώνευσης, πλούσιο σε μακροστοιχεία και ιχνοστοιχεία απαραίτητα για την σωστή ανάπτυξη των φυτών. Ο βασιλικός (*Ocimum Basilicum* L.) είναι ένα από τα πιο σημαντικά φαρμακευτικά και αρωματικά φυτά (MAPs) με μεγάλη οικονομική σημασία, το οποίο χρησιμοποιείται ευρέως στην Ιταλική και Ελληνική κουζίνα. Για να διερευνήσουμε την επίδραση του χωνεμένου λύματος ως εναλλακτικού λιπάσματος στην ανάπτυξη του βασιλικού, πραγματοποιήσαμε ένα διετές πείραμα. Το πείραμα αποτελούνταν από τέσσερις πειραματικές εφαρμογές: χημικό λίπασμα (F), χωνεμένο λύμα (D), συνδυασμός χημικού λιπάσματος και χωνεμένου λύματος (1:1, FD), και φυτά χωρίς λίπανση «μάρτυρας» (C). Μετρήθηκαν σημαντικοί παράγοντες που συνδέονται με την ανάπτυξη των φυτών, όπως ύψος φυτών, ολική βιομάζα φυτών (TB), παραγωγή βιομάζας φύλλων (LP), δείκτης συγκέντρωσης χλωροφύλλης (CCI), παραγωγή και σύσταση αιθέριων ελαίων. Μετά την συγκομιδή πάρθηκαν δείγματα εδάφους για τον υπολογισμό των βαρέων μετάλλων και των θρεπτικών στο έδαφος καθώς και της συγκέντρωσης παθογόνων μικροοργανισμών στο έδαφος και στα φύλλα του βασιλικού. Τα αποτελέσματα έδειξαν ότι η εφαρμογή FD αύξησε σημαντικά την TB (68.2 g $plant^{-1}$) και LP (52.7 g $plant^{-1}$), ενώ παράλληλα έδειξε την υψηλότερη παραγωγή αιθέριου ελαίου ανά εκτάριο. Η χρήση χωνεμένου λύματος αύξησε την συγκέντρωση των απαραίτητων μακροστοιχείων, φωσφόρου (P) και καλίου (K) κατά 68.5% και 134.4%, αντίστοιχα, ενώ σημαντική αύξηση παρατηρήθηκε και την οργανική ουσία του εδάφους κατά 54.7%. Η υψηλότερη συγκέντρωση αιθέριων ελαίων παρουσιάστηκε στα φυτά μάρτυρες. Αξιοσημείωτο είναι ότι οι διαφορετικές εφαρμογές λίπανσης επηρέασαν την σύνθεση των δευτερογενών μεταβολίτων. Συγκεκριμένα, τα φυτά που δέχθηκαν χωνεμένο λύμα παρουσίασαν σημαντικές αυξήσεις στην σύνθεση σεσκιτερπενίων και ειδικότερα της γερμακρένης D και της β-ελεμένης. Η χημική λίπανση αύξησε την σύνθεση της μεθυλευγενόλης και της β-φαρνεσίνης, οι οποίες συνδέονται με την άμυνα των φυτών απέναντι σε βιοτικούς κινδύνους και την αναπαραγωγή. Οι μάρτυρες έδειξαν σημαντική αύξηση στην σύνθεση των μονοτερπενίων και ειδικότερα της λιμονένης, ευγενόλης, και λιναλοόλης, οι οποίες συνδέονται με την άμυνα των φυτών απέναντι σε αβιοτικούς κινδύνους, όπως την καταπόνηση λόγω κρύου και ξηρασίας, ενώ διαθέτουν και αυξημένη αντιβακτηριακή, αντιμυκητιακή και αντινεοπλασματική δραστηριότητα. Επομένως, η έλλειψη λιπάσματος αύξησε την δημιουργία τερπενίων που συνδέονται με την αντιμετώπιση καταπονήσεων και αβιοτικών κινδύνων, η χρήση χημικού λιπάσματος οδήγησε στην αύξηση της σύνθεσης τερπενίων που συνδέονται με βιοτικούς κινδύνους και την αναπαραγωγή, η χρήση χωνεμένου λύματος την σύνθεση σεσκιτερπενίων, και οι συνδυασμένη λίπανση οδήγησε στην παραγωγή αιθέριου ελαίου ουδέτερης σύνθεσης. Αξιοσημείωτο είναι ότι *Salmonella* spp. δεν εντοπίστηκαν ούτε στο έδαφος αλλά ούτε στα φύλλα των φυτών. *Enterococcus faecalis* και *Escherichia coli* εντοπίστηκαν σε μικρές ποσότητες στο έδαφος (< 9.1 cfu g^{-1}). Εν αντιθέσει η συγκέντρωση *Enterococcus faecalis* στα φύλλα των φυτών που λιπάνθηκαν με χωνεμένο λύμα ήταν σχετικά υψηλή (91est στα D φυτά και 73 est cfu g^{-1} στα FD φυτά), οριακά κάτω από το όριο των 100 cfu g^{-1}, για φρέσκα λαχανικά και φρούτα έτοιμα προς βρώση, ενώ οι συγκεντρώσεις *E. coli* παρέμειναν χαμηλές στα φύλλα των φυτών (< 9.1 cfu g^{-1}).

Chapter 1: Introduction

1.1 Anaerobic digestion process, systems, and reactor types

1.1.1 Anaerobic digestion process

Anaerobic digestion (AD) is a naturally occurring process during which organic matter biodegrades, producing a mixture of flammable and non-flammable gases collectively known as biogas (Meenakshisundaram et al., 2021; Sihlangu et al., 2024a). AD not only produces biogas, a renewable energy source, but simultaneously sustainably treats agricultural waste. The process involves the decomposition of organic matter in anoxic conditions by a consortium of diverse microorganisms (Neri et al., 2023). AD is divided into four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Methanogenesis is considered the rate-limiting step in AD for easily degradable substrates because methanogenic archaea are sensitive to environmental changes and grow slowly (Wang et al., 2023). However, when lignocellulosic substrates are employed, hydrolysis becomes another limiting factor, due to the slow rate of lignocellulose degradation (Woźniak et al., 2025).

At the beginning of the process, hydrolytic extracellular enzymes degrade large organic polymers into simple soluble monomers. Hydrolysis converts proteins, lipids, and carbohydrates to amino acids, fatty acids, and sugars. In acidogenesis, these soluble monomers (monosaccharides, fatty acids, and amino acids) are mainly converted to volatile fatty acids (VFAs) and, to a lesser extent, to alcohols, hydrogen (H_2), carbon dioxide (CO_2), and ammonia (Rezapoor and Rahimpour, 2024). Acetogenesis transforms the higher VFAs (propionate, butyrate, etc.) and alcohols into acetate, hydrogen, and carbon dioxide. Finally, methanogenic archaea convert acetate, CO_2, and H_2 into biogas, which primarily comprises methane and carbon dioxide (Agregán et al., 2022; Dianat et al., 2024).

There are several methanogenic pathways, but the two main ones are the acetoclastic and the hydrogenotrophic. Acetoclastic is usually the dominant path with its contribution ranging between 60 and 70%, while hydrogenotrophic methanogenesis typically contributes between 30 to 40% (Chang et al., 2024; Pan et al., 2016). However, elevated ammonia levels can seriously disrupt the process and shift the pathway from predominantly acetoclastic to hydrogenotrophic (Bucci et al., 2024; Chen et al., 2016).

The efficiency and stability of the AD process depend on several key factors, including pH, temperature, C:N ratio, organic loading rate (OLR), and hydraulic retention time (HRT) (Jameel et al., 2024). The optimal pH for AD is between 6.8 and 7.2 (Cioabla et al., 2012), although some researchers claim that the system can tolerate pH increases up to 8.0 (Hmaissia et al., 2025). pH values between 5.5 and 6.5 are desirable for the acidogenesis stage, while values ranging between 6.5 and 7.5 are optimum for methanogenesis (Ishak et al., 2022). However, pH values lower than 5 can limit the process efficiency due to the higher rate of methanogenic archaea decay (Janesch et al., 2021; M. Sun et al., 2020). Moreover, pH increases over 8.0 can lead to severe elevations in free ammonia (NH_3) concentrations, leading to severe inhibition of the AD process, and even complete process failure (Shi et al., 2024).

AD can operate under mesophilic (ranging from 20 °C to 35 °C), thermophilic (ranging from 55 °C to 80 °C), and psychrophilic (< 15 °C) conditions (Alblooshi et al., 2025). Mesophiles are found in ambient temperatures, thermophiles are predominantly found in hot springs, while psychrophiles have been observed in frigid environments such as polar regions or in the deeper and colder parts of the ocean (Alblooshi et al., 2025). A shift from mesophilic to thermophilic conditions requires a period of microbial adaptation to the new operating conditions to avoid decreases in biogas production (Westerholm et al., 2018).

C:N is a crucial factor for an efficient biodegradation process. Substrates with a low C:N ratio have a high concentration of nitrogen, which leads to high ammonia accumulation and process inhibition. Conversely, substrates with a high C:N ratio present high carbon content but inadequate nitrogen for microbial growth, leading to lower VFA production (Tg et al., 2022). The optimum C:N ratio for AD ranges from 20-30, while the optimum ratio is 25. C:N ratio is an important index highlighting the availability of nutrients for the microbial consortia performing the biodegradation process. Since C:N ratios vary depending on the substrate, combining two or more substrates (co-digestion) can facilitate the achievement of a more favorable ratio (Surra et al., 2019).

OLR is another important parameter. It has been described as the amount of organic matter introduced in the digester per volume, per day, and plays a key role in biogas yield and composition (Gautam et al., 2022; Nkuna et al., 2022). High (OLR) can cause excessive VFA accumulation and pH reduction, leading to severe inhibition of the microbial consortia's activity, disruption of the process, and potential system failure (Z. Li et al., 2024). Low OLRs are usually preferable for sufficient substrate degradation.

Hydraulic retention time (HRT) refers to the average time the substrate remains in the digester and is defined as the ratio of the digester's volume divided by the daily feeding rate (Srisowmeya et al., 2020). HRT is a key parameter in AD, and coupled with OLR, is crucial for the AD system's stability. Longer HRTs are required to enhance substrate biodegradation (Ruile et al., 2015), while the optimum HRT depends on the substrate composition and the type of the digestion system.

1.1.2 Digestion systems

Likewise, the digestion system depends on the characteristics of the substrate. Specifically, high moisture feedstocks (> 85%) are usually treated via wet digestion. Such feedstocks are sewage sludge and animal manure/slurry. In a wet system, adequate contact between the feedstock and the microbial community is ensured via mechanical agitation, gas sparging, or recirculation of digestate (Uddin and Wright, 2023). Mechanical agitation also offers other benefits, such as feedstock uniformity and increased process kinetics (Z. Zhang et al., 2023). Mechanical agitation is regarded as the most efficient and economically advantageous methodology for wet AD, as it shortens the start-up period and increases the stability and efficiency of the process (Ghanimeh et al., 2012). In contrast, dry digestion is suitable for substrates with high total solids (TS) concentration (15%-40%), such as agricultural residues and livestock manures (Zhongzhong Wang et al., 2023).

There are two categories of digesters: single-stage and two-stage. In a single-stage digester, all of the steps of AD are performed in a single digester. In a two-stage digester, the first two steps (hydrolysis and acidogenesis) and the latter two (acetogenesis and methanogenesis) are performed in different digesters, with each digester having different operational conditions, optimal for different microbial groups (Zheng and Li, 2024). In particular, the first digester facilitates the hydrolysis and acidogenesis process, while the second facilitates the acetogenesis and methanogenesis process. In order to accelerate the first two steps of AD, the pH in the first digester is lowered (approximately to 5-6), and temperatures are elevated to approximately 50-55 °C. In the second digester, the pH is elevated to 7.0-8.0, while the temperature is lowered to

30-40 °C, to enhance the acetogenesis and methanogenesis processes (Zheng and Li, 2024). Two-stage AD can also be used for the production of hydrogen (H_2) during the first stage and methane during the second (Zheng and Li, 2024).

Regarding wet digestion, there are several reactor designs, such as continuously stirred tank reactors (CSTR). These reactors are suitable for liquid waste, slurries, and organic waste with a high moisture content. CSTRs' continuous mixing of the feedstock ensures uniform conditions, especially temperature, in the reactor and adequate suspension of the solids, making them easier to operate and scale up from lab to industrial scale (Cherkasov et al., 2023). However, CSTRs are prone to microorganism washout and are considered less efficient compared to other reactor types, such as plug flow, at short HRTs (Balsamo and Montagnaro, 2018).

1.1.3 Digestor types

Upflow anaerobic sludge blanket (UASB) reactors are used to treat organic wastewaters with low suspended solid content. UASBs are especially effective when treating highly biodegradable substrates. This enables the formation of a dense, granular sludge blanket, which is imperative to ensure functionality and stability of the process (Srivastava et al., 2021). The main advantages of USAB are that they can handle high OLRs in short HRTs, while having low nutrient and energy requirements. However, they require extended start-up periods and are often met with corrosion and maintenance issues (Daud et al., 2018).

Plug Flow digesters are designed to treat feedstocks with relatively high total solids (11-14%) and move the feedstock in a single “plug”, hence the name (Cantrell et al., 2008; Mutungwazi et al., 2018). They are typically shaped like a tube, and the organic material moves through the tank, with new materials pushing existing materials towards the outlet, negating the need for mixing (Mutungwazi et al., 2018). Other designs include Fixed Film digesters, where microorganisms grow on a plastic or any other inert material surface, and anaerobic contact reactors (ACRs), which are similar to CSTRs, but the effluent passes through a solid-liquid separation unit, and the recovered microbial solids are reintroduced into the digester (Banerjee et al., 2022; US EPA, 2016).

2.1 Pretreatment for lignocellulosic substrates

2.1.1 Physical pretreatment

Physical pretreatment aims to increase the substrate's surface area, reduce its viscosity, and improve contact between substrate and microorganisms. Milling or grinding reduces the substrate’s particle size, decreases cellulose crystallinity, increases surface area and porosity, thus reducing digestion time and improving substrate biodegradation (Di Domenico et al., 2025).

Particle size reduction has been employed by several researchers aiming to enhance lignocellulose biodegradability with considerable achievements. Akhlisah et al. (2021) reported significant disruptions of the surface area and an increase in the volume of palm oil biomass when milled or ground prior to digestion. Hence, the access area for the hydraulic enzymes increased significantly, resulting in enhanced substrate degradation. Jomnonkhaow et al. (2021) stated that mechanical pretreatment can enhance substrate biodegradability by releasing the constituents of plant cells, while new surface area is formed, which is more easily accessible to the microbial community. Mechanical pretreatments also increase bacterial activity, morphology, and substrate cell solubility (Mothe et al., 2024). However, some researchers have reported limited results with mechanical pretreatment (Tsapekos et al., 2016); hence, more advantageous and efficient methods have been explored.

2.1.2 Chemical pretreatment

Chemical pretreatments offer the most promising results and are commonly applied to lignocellulosic biomasses because they effectively reduce cellulose crystallinity and enhance lignin removal, while they can increase substrate biodegradability by up to 88.3% (Mohammad Rahmani et al., 2022). However, chemical pretreatments are considered relatively expensive and tend to produce toxic substances capable of hindering the AD process (Mohammad Rahmani et al., 2022).

Alkali pretreatment is the most effective and commonly employed chemical treatment, substantially reducing cellulose crystallinity, removing lignin, and increasing surface area and porosity (Bochmann, 2019; Shakeel et al., 2024). Alkaline ions break ester-ether bonds between lignin and polysaccharides and weaken hydrogen bonds between hemicellulose and cellulose. Common alkalis such as $Ca(OH)_2$ and NaOH have shown significant increases in methane yield (e.g., 11.99% with $Ca(OH)_2$, 155% with NaOH) (Li et al., 2024). Moreover, alkalis combined with moderate temperatures (55 °C) have been shown to increase biomethanation while decreasing the risk of forming substances such as furfural and 5-HMF, which are toxic to the anaerobic microbes.

Acids can enhance lignocellulosic biodegradability by hydrolyzing the glycosidic bonds in hemicellulose, increasing saccharification efficiency, and disrupting the structure of the recalcitrant biomasses. Thereby, making cellulose and the remaining components more accessible for subsequent enzymatic hydrolysis (Lv et al., 2024). Dilute acids are preferable in comparison to concentrated acids due to their cost-effectiveness and low corrosive potential; however, high temperatures are imperative to ensure pretreatment efficiency. In contrast, strong acids are more corrosive, require neutralization, and are more difficult to handle and recover, but are considered more effective (Panigrahi and Dubey, 2019; Woźniak et al., 2025). Specifically, using strong acids (e.g., sulfuric, nitric, and hydrochloric) can lead to the formation of inhibitory by-products such as H_2S, furfural, and phenols (Bochmann, 2019; Yu et al., 2022). Combining acids or alkalis with heat (e.g., 10% NaOH at 100 °C) has been shown to further increase the substrate's degradability and methane potential, while electrochemical methods combined with alkali can enhance sludge floc breakdown (X. Li et al., 2024).

2.1.3 Biological pretreatment

Biological pretreatments use fungi, bacterial strains, or enzymes to enhance lignocellulosic and other recalcitrant biomass degradation (Zabed et al., 2019). Specifically, white-rot fungi (WRF) are particularly effective at delignification, secreting hydrolases and ligninolytic enzymes. Rice straw treated with WRF showed increased methane production, with methane yields doubling (Saritha et al., 2012). According to Saritha et al. (2012), these increases were attributed to the enhanced lignin degradation of the rice straw. However, this process is time-consuming and can last from weeks to months. Therefore, this process cannot be implemented in commercial biogas power plants due to the time required for the fungi to break down the substrate (Zabed et al., 2019). Hence, diversification of the fungal consortia is used to overcome the disadvantages of pure cultures by providing diverse enzymatic capabilities (Thirumalaivasan et al., 2024). According to Li et al. (2024), methane production significantly improved when the lignocellulose in municipal solid waste (MSW) was treated using the aforementioned methodology.

Another biological method is enzymatic pretreatment and involves using microbial enzymes to enhance pre-treatment. Specifically, laccases and peroxidases are often used as lignin degraders. Li et al. (2024) reported increased methane yields when corn stover and switchgrass were treated with a combination of laccases and peroxidases. Enzyme pre-treatment is more effective on lignocellulosic biomass and sludge, but less effective in substrates with high natural hydrolysis rates like food waste (Hosseini Koupaie et al., 2019). In addition, combined pre-treatment methods, such as combining biological with physical or chemical pre-treatments, are considered highly promising due to their synergistic effects, especially in co-digestion systems, ensuring a more balanced environment for the microbial consortium and ample quantities of nutrients for the microbes (Kumar Prasad et al., 2024).

2.1.4 Thermal pretreatment

The aim of pretreating recalcitrant substrates with heat is to break down their hydrogen bonds and rigid structures. Application of high temperatures (ranging from 160 to 190 °C) for 20-30 minutes has been shown to substantially enhance biogas production of lignocellulosic biomass. Moreover, studies have demonstrated a 30% increase in biogas production when the temperature was increased to 120 °C and a 92% increase at 170 °C (Nayeri et al., 2024). However, temperatures exceeding 160 °C can dissolve hemicellulose and lignin, potentially forming phenolic compounds that are capable of inhibiting the growth of the microbial populations (Gladysheva, 2025; Gossett et al., 1982).

Freeze-thaw is another method used to improve sludge biodegradability, with slow freezing exhibiting better results than rapid freezing. In particular, slow freezing disrupts the sludge cell walls, promotes the formation of crystals, and significantly reduces the water content in the sludge (Y. Wu et al., 2022). Microwave pre-treatment generates energy through an electromagnetic field, creating friction between molecules, while dipole rotation promotes rapid and uniform heating, enhancing sludge dissolution and methane formation (Kumar and Verma, 2025). Ultrasound pre-treatment increases methane production by approximately 31-84%, soluble chemical oxygen demand (SCOD) by approximately 30%, and reduces volatile solids and sludge volume, albeit it is highly energy-intensive (Nayeri et al., 2024). Moreover, this method is mainly used for sludge pretreatment and utilizes cavitation to disrupt the cell walls. Specifically, it employs high frequencies of 50-40 kHz to create bubbles that eventually burst, creating shearing forces (Mitraka et al., 2022a).

2.1.5 Additives

The use of conductive additives, including biochar, magnetite, and graphene, has been shown to enhance interspecies electron transfer (IET) and direct interspecies electron transfer (DIET) between microbes, improving methane generation. DIET acts as an alternative to interspecies hydrogen/formate transfer (IHT/IFT). Studies have shown that adding 1.0 g L^{-1} graphene increases methane yield by 25% and methanol degradation by 29% (Lin et al., 2017). Magnetite can also reduce VFA accumulation, increase methane production, and enrich bacterial and archaeal genera. Non-carbon-based materials like ferrosoferric oxide have also shown promising results in improving DIET between the microbial communities (Kong and Zhang, 2023; Park et al., 2018).

Functional Microbial Additives (Bioaugmentation and Enzymes) are enrichment agents that are introduced to the anaerobic reactor, aiming to improve performance and enhance biomethanation (Das et al., 2023; Nzila et al., 2016). Moreover, they increase the resistance of AD microbes to toxic substances such as organic acids and ammonia and enhance substrate decomposition. Lignocellulolytic microbial consortia have been shown to increase biogas yield from fiber-rich swine manure up to 55% (Tuesorn et al., 2013), while bioaugmentation can effectively increase methane production and alleviate ammonia inhibition (Nzila et al., 2016). However, bioaugmentation microorganisms and enzymes must not be added above certain threshold levels, because they can become toxic to the process, while their high cost can be an obstacle for large-scale applications (Das et al., 2023; C. Li et al., 2024; Nzila et al., 2016a).

Another methodology used to improve environmental conditions for the microbial community within the digester is the addition of Trace Elements (TEs) (Li et al., 2024). TEs are metals (e.g., Mn, Fe, Co, Cu, Mo, Ni, Se, W) vital for microbial metabolism in low concentrations, while they can act as inhibitors in excessive quantities. Their main role is to promote the conversion of VFAs, H_2, and CO_2 to methane. Moreover, they regulate the rate of VFA production and participate in the creation of metalloenzymes, which are crucial in microbial metabolism. The amount of TEs varies significantly with substrate type, digestion mode, and operating temperature. Particularly, thermophilic digestion typically requires higher TE concentrations, and mesophilic digestion requires lower concentrations. Substrates with low TE concentrations, such as kitchen waste and lignocellulosic biomass, are more prone to TE deficiency than livestock manure. The bioavailability of TEs is crucial, as some may be present in non-bioavailable forms. Chelating agents like EDTA and EDDS can regulate TE bioavailability by increasing water-soluble and exchangeable fractions. However, excessive TE addition can lead to toxic effects, while the adverse environmental impact of ligands must also be taken into consideration prior to addition (Pires et al., 2025; Zhi Wang et al., 2023).

Biochar has the capacity to aid anaerobic digestion and mitigate toxicity issues in the reactor. It is produced from biomass pyrolysis and is rich in functional groups, while having a large surface area, porosity, and conductivity. It functions by adsorbing toxic substances (e.g., ammonia), enhancing DIET, and immobilizing microorganisms. Biochar's buffering capacity can also reduce VFA concentrations. It can activate hydrolytic bacteria and enzymes, which increase AD efficiency. However, biochar application in large-scale projects is limited because it is difficult to reuse and relatively expensive (Nie et al., 2024).

2.1.6 Recirculation

Recirculation involves reintroducing digestate, sludge residue, or leachate to the reactor. It also facilitates the distribution of key nutrients, such as nitrogen, phosphorus, and microorganisms throughout the digester, promoting a more stable and balanced environment. It prevents the solids from settling and ensures better contact between microorganisms and substrate (Li et al., 2024). The reuse of the liquid effluent helps the process to become more sustainable, reduces the need for external freshwater, which is a major advantage in water-scarce regions, while enhancing the microbial population by reintroducing washed-out microorganisms (Shoshaa et al., 2024). Recirculating the digestate reintroduces a high concentration of active, acclimated microorganisms, including methanogenic archaea, back into the digester. This can speed up the startup of a new digester and enhance the stability of an existing one, particularly by inoculating the fresh substrate with a healthy microbial population (Li et al., 2024). However, while beneficial, recirculation must be carefully managed, especially when digesting nitrogen-rich substrates like livestock manure and food waste. The breakdown of proteins and other nitrogenous compounds produces ammonia (NH_4^+) (Yang et al., 2025). A moderate concentration of ammonia is necessary for microbial growth, but high concentrations can easily become toxic. The highly toxic form of ammonia is free ammonia (NH_3), which can easily diffuse into microbial cells and disrupt their internal pH balance, leading to cell death. This can severely inhibit methanogenesis, causing a drop in biogas production and potential AD failure (Guo et al., 2023).

2.1.7 Microaeration

Microaeration is a technique that involves the controlled supply of small volumes of air or oxygen (typically 0.005-5 L $O_2/L_{reactor}$/d) to the anaerobic reactor (Castro et al., 2023; Magdalena et al., 2022). Although oxygen typically inhibits methanogenesis, microaeration can increase hydrolysis rates, mitigate toxicity phenomena from high concentrations of hydrogen sulfide (H_2S), and improve process stability at high OLRs (Krayzelova et al., 2015). Microaeration primarily balances the composition and activity of hydrolytic, acidogenic, and methanogenic archaea. It encourages the formation of diverse and highly active hydrolytic and fermentative bacterial communities, particularly increasing Firmicutes, which are linked to high hydrolytic rates and extracellular enzyme production (Fu et al., 2016; Zhou et al., 2021).

3.1 Feedstock for biogas production

Feedstocks for AD are organic materials that can be converted into methane. They range from biodegradable wastewater to complex high-solid waste (Tshemese et al., 2023). The feedstock type directly influences biogas composition, methane yield, process stability, and the overall economic viability of a biogas power plant.

Feedstock type, substrate particle size, and the presence of easily degradable sugars, amino acids, and fats, as well as the cell structure of the feedstock (e.g., lignocellulosic), are very important in estimating the substrate's biodegradability and hence its methane yield potential. Therefore, the greater the digestibility of the feedstock, the higher the biogas yield (Kupryaniuk et al., 2025)

3.1.1 Agricultural wastes and crop residues

Agricultural residues constitute one of the largest and most accessible sources of biogas feedstock globally. These materials include animal manure, skin and carcasses, fruit wastes, and crop residues such as straw, bagasse, husks, and various other agricultural and agro-industrial processing wastes (Adetunji et al., 2023; Ngabala and Emmanuel, 2024a).

Livestock manures constitute the largest volume of organic waste in the agricultural sector, containing a high concentration of nutrients essential for plant production (Cucina, 2023). Consequently, most farmers use it as a soil improver and as an inexpensive organic fertilizer (Köninger et al., 2021). However, animal manure can also become a source of pollution by introducing heavy metals and pathogens to the soil (Qi et al., 2023).

Vast quantities of waste in the form of manure are produced each year in the EU, since the EU is the third largest beef and veal producer and the second pork producer behind only China (Akamati et al., 2022; Vinci, 2022). The annual manure production (in dry matter) from the EU countries is projected to reach between 83 and 122 Mt y^{-1} by 2030. The majority of this waste will stem from cattle farming (63 to 88 Mt y^{-1}), with swine and poultry manure following with 18-23 and 8-11 Mt y^{-1}, respectively. Moreover, the total energy potential from animal manure by 2030 is expected to range between 670 and 890 PJ y^{-1}, comprising 320 to 420 Mt y^{-1} from swine manure, 280-390 Mt y^{-1} from cattle manure, and 60-80 Mt y^{-1} from poultry (Meyer et al., 2018). Scarlat et al. (2018) reported that the theoretical biogas potential from animal manure in Europe is estimated at 26 billion m^3 of biomethane (23 billion m^3 from the EU), with a more conservative scenario placing the European biogas potential at 16 billion m^3.

Another abundant source of feedstock for anaerobic digestion is straw. Straw is the residual product remaining in the fields after harvesting cereals such as rye, wheat, rice, maize, and sorghum (Ammenberg and Feiz, 2017). According to Passoth and Sandgren (2019), wheat straw comprises approximately 35-39% cellulose, 23-30% hemicellulose, and 12-16% lignin, while rice straw comprises 29-35% cellulose, 12-29% hemicellulose, and 17-19% lignin. The net cumulative methane production (NCMP) is estimated at 162 ml CH_4 g^{-1} VS for rice straw and 266 ml CH_4 g^{-1} VS for wheat straw (Dai et al., 2020). The energy potential from straw is high, while the procurement cost is relatively low, especially compared to energy crops.

The three main crops cultivated globally are wheat, maize, and rice. Collectively, these three crops provide 60% of the world's calories, with almost half of the world's population relying on rice for most of their daily calories (Shi et al., 2022). In Greece, the cultivated rice area is estimated to be approximately 20,000 to 30,000 hectares, while head rice production ranges between ~180,000 to ~255,000 tons annually (Kalaitzidis et al., 2025). The high straw production from rice cultivation (each kg of rice grain produces 1.4 to 1.5 kg of straw) suggests that there are ample quantities of rice straw that can be utilized for methane production (Singh and Patel, 2022). Currently, however, rice straw is customarily burned in the fields post-harvest, causing health issues to the local population and polluting the air with hazardous chemical compounds and particles (Dinh et al., 2024). Therefore, utilization of rice straw for methane production can also facilitate better living standards for the rural population residing adjacent to paddy fields.

Other significant lignocellulosic residues stemming from the agro-industrial sector are the dry pulpy residues from sugar cane and sugar beet processing, collectively known as bagasse. Bagasse is a highly promising substrate for biogas production. However, due to its low availability and difficulty in long-term storage (it has a high water content and spoils easily) (Ammenberg and Feiz, 2017), it is customarily used as fuel (Agarwal et al., 2022).

3.1.2 Municipal and food wastes

According to Eurostat, in 2022, the average municipal solid waste (MSW) production per person in the EU was 513 kg $year^{-1}$ (Eurostat, 2022). In 2021, Asian countries produced 1 Mt of MSW per day, projected to reach 1.8 Mt day^{-1} in 2025. Such huge quantities of waste need to be treated sustainably in order to avoid health risks stemming from pathogens residing in human excreta. Moreover, the treatment of such wastes reduces odors, and post-treatment, they can be used in agriculture as organic fertilizers. MSW comprises domestic and commercial wastes (Sharma and Jain, 2020) and primarily contains easily degradable carbohydrates. Therefore, MSW is an ideal substrate for biomethane production (Kobayashi et al., 2012).

Anaerobic digestion of sewage sludge not only produces renewable energy but simultaneously stabilizes the sludge, making it safe for disposal (Pilarska et al., 2024a). Energy production from MSW can cover the energy requirements of the sludge processing facility while treating a health-risk-associated organic mixture (Dąbrowska and Masłoń, 2020). Hence, anaerobic digestion can facilitate the conversion of wastewater treatment plants into energy-positive facilities, enabling them to contribute to local energy grids (Pilarska et al., 2024b).

3.1.3 Industrial wastes

Industrial wastes encompass a myriad of unwanted by-products from the agro-industrial sector, such as waste from paper processing, slaughterhouses, grain mills, dairies, poultry, abattoirs, fish, and vegetable processing (Achinas et al., 2017). Traditionally, these wastes were managed by composting them in landfills, dumping, or incinerating them (Adetunji et al., 2023). However, these wastes are rich in proteins, fats, and carbohydrates, making them ideal substrates for conversion into biogas through AD. For instance, research has shown that co-digestion of brewery spent grain with sewage sludge leads to improved biogas yields and kinetics (Szaja et al., 2020).

Moreover, organic wastes from meat processing facilities are protein-rich and have high biogas potential. These wastes stem from cutting and handling the carcasses, poultry de-feathering, moldy meat or carcasses with microbial or other infestations, and any products damaged during transportation. Slaughterhouse and abattoir wastes comprise animal blood, fat, paunch, and other nitrogen-rich by-products (Mofijur et al., 2021; Ngabala and Emmanuel, 2024b). Meat processing wastes, especially those derived from the poultry industry, are rich in organic nitrogen (35.5-50 g kg^{-1}) and relatively low in carbon, hence presenting a low carbon to nitrogen (C:N) ratio (7.9-9.8). Their low C:N ratio is unfavorable for AD and therefore requires the addition of another feedstock rich in carbon to reach the optimum C:N ratio of 20 to 30 (C. Liu et al., 2022; Rajlakshmi et al., 2023).

Fish processing facilities produce billions of tons of high-energy, easily degradable waste annually. Due to their high protein, lipid, and organic content, fish wastes are ideal for biogas conversion. In particular, research has shown that anaerobic digestion of salmon smolt hatchery sludge in a CSTR system resulted in high biogas production with a methane content of 59-61% and net energy generation of 43-47 MWh $year^{-1}$ (Bücker et al., 2020).

Other studies have reported that sludge from pulp and paper mills can be successfully used as substrate for biogas generation. Pulp and paper mill sludges contain large organic components that are easily degradable. Moreover, each ton of paper produces approximately 1 m^3 of sludge. Paper sludge is a highly carbonic organic substrate, requiring co-digestion with a nitrogen-rich material, such as cow manure or swine manure (Priadi et al., 2014). However, it is recalcitrant in nature, thus requiring pretreatment to enhance its biodegradability, with thermal and thermochemical pretreatments significantly improving its biogas production potential (Bayr et al., 2013).

3.1.4 Aquatic biomass

Feedstocks such as water hyacinth, micro and macro algae, and seaweeds are also suitable for bioenergy production. These feedstocks have easily hydrolysable sugars, contain low lignin content, and do not compete with land resources used in arable food crop production (Khan et al., 2018).

Water hyacinth leaves and stems can be utilized for biogas production, particularly when co-digested with animal manure, as they exhibit a high cellulose content (Mulu et al., 2025). Results showed that the highest biogas and methane yield was derived from the fresh stems and reached 597 and 326 L kg^{-1} VS, respectively, while the digestate produced from their AD is considered a valuable organic fertilizer.

Algae constitute a huge and diverse group of photosynthetic organisms, from simple unicellular microalgae (3-10 μm), such as diatoms, to large macroalgae or kelps (up to 70 m long), growing as fast as 50 cm day^{-1}. Algae grow wherever there is light to photosynthesize, while sea algae form the backbone of the marine food chain (El Gamal, 2010). The morphology of macroalgae (or seaweeds) is similar to terrestrial plants, but their biochemical constituents are considerably different. Their water content is particularly high (70-90%), absorbing the nutrients directly from the water due to a lack of a vascular system (Cesário et al., 2018; Kundu et al., 2024).

Farmed blue mussels (*Mytilus edulis*) can also be used for biogas production (Ammenberg and Feiz, 2017). Blue mussels are among the most common mollusks in the Baltic Sea. Mussel farming in the Baltic Sea spread due to the eutrophication appearing in the area. Mussels are filter feeders and thus remove nutrients from the sea while feeding. However, they are considered very small for human consumption. Therefore, their digestion for biogas production has been explored as a viable alternative (Nkemka and Murto, 2013; Wollak et al., 2018).

3.1.5 Specialized feedstocks

In Tanzania, sisal plants are being farmed for the production of durable fibers. However, only 3 to 6% of the plant is utilized, and the rest is discarded. Sisal waste is a lignocellulosic by-product suitable for anaerobic digestion. Due to its recalcitrant nature, most studies suggest that pretreatment is imperative prior to digestion (Konyo et al., 2023; Mshandete et al., 2004).

3.2 Feedstock characteristics

3.2.1 Proteins, lipids, and carbohydrates

All forms of organic biomass, containing essential macronutrients such as proteins, lipids, and carbohydrates, are suitable for biogasification (Ngabala and Emmanuel, 2024b). The three main macronutrients: lipids, carbohydrates, and proteins influence both the biogas yield and the methane concentration in the final gas mixture.

Lipids are categorized according to their state at room temperature, appearing either as solids (waxes and greases) or liquids (oils). Fats can be found in dairy residues, slaughterhouse wastes, industrial lipids, sterols, and fat-soluble vitamins, while lipids exist in all biological organic matter (Khan et al., 2021). Lipids have a greater methane potential because they contain a higher density of carbon and hydrogen atoms per molecule than other macromolecules (Ngabala and Emmanuel, 2024). Consequently, they produce higher quantities of biogas but require more time to biodegrade. Conversely, proteins and carbohydrates convert more quickly, but generate less biogas (Ohemeng-Ntiamoah and Datta, 2018)

Carbohydrates are the most rapidly degraded compounds in AD, often leading to the accumulation of VFAs. This accumulation can cause a significant decrease in the reactor's pH, thereby inhibiting the AD process. However, carbohydrates from recalcitrant organic materials are difficult to hydrolyze, necessitating pretreatment prior to digestion. Otherwise, they can hamper the process, increasing the retention time required to process the feedstock (Hasani Andevari et al., 2025).

On the other hand, proteins break down into amino acids and provide valuable nitrogen to the system for microbial growth. Moreover, some of the nitrogen is transformed into ammonia, which is crucial for maintaining the system's buffering capacity and keeping the pH within optimal ranges. Nevertheless, substrates with an excessive nitrogen content can lead to the formation of inhibitory concentrations of free ammonia, which is highly toxic to methanogens.

3.2.2 Role of carbon and nitrogen

Nitrogen is used by the microbes to synthesize their proteins, allowing for rapid cell and population growth. Nitrogen is an essential component of amino acids, which are the building blocks of proteins, and nucleic acids like DNA and RNA (Koch and Sessitsch, 2024). Moreover, nitrogen, usually in the form of ammonia, is used by the anaerobic microbes to synthesize the proteins and nucleic acids needed for their own cells (Edwards et al., 2024). Therefore, ammonia, apart from acting as a regulator of the system's pH, increasing the system's buffering capacity, simultaneously plays a critical role in the synthesis of proteins essential for the anaerobic microbial population.

Carbon is the fundamental building block for cells and, therefore, invaluable for the microbial community. It provides energy and plays a key role in the metabolic activity of the microbes (Saini et al., 2011). Bacteria use carbon to synthesize fats, carbohydrates, and nucleic acids, while they can utilize both organic and inorganic carbon sources (Bonnet et al., 2020). Additionally, some researchers have noted that activated carbon can increase the electron transfer between microbial species, enhancing methane production (F. Wu et al., 2022; Xie et al., 2019).

4.1 Pretreatment methods for rice straw

Rice straw is one of the most abundant agricultural residues globally. As a lignocellulosic biomass, it is highly carbon-rich with substantial potential for biogas production. However, its recalcitrant nature stems from a complex structural matrix of cellulose, hemicellulose, and lignin. As a result, only 10 to 20% of its polysaccharides are converted to monosaccharides without pretreatment (Yoon et al., 2014). Pretreatment aims to break down the complex cellulose, hemicellulose, and lignin matrix, enabling cellulases to access and hydrolyze rice straw's celluloses and hemicelluloses.

4.1.1 Biological pretreatment methods

Biological pretreatment of feedstock for AD has attracted interest because it requires less energy input compared to physical pretreatment and is less costly than chemical pretreatment.

Several biological methods have been investigated for rice straw pretreatment. In a study performed by Shetty et al. (2020), fiber-digesting anaerobic fungi were used to degrade rice biomass. The purpose was to enhance the biodegradation of rice straw by adding the fiber-digesting fungus, *Orpinomyces joyonii*, to the inoculum. Adding this fungus increased the average daily biogas and methane yield by 25% and 38% respectively. Moreover, the reduction in volatile solids, cellulose, and hemicellulose appeared to increase by 68%, 82%, and 97%, respectively.

Kumar et al. (2021) evaluated the potential of using isolated bacterial strains to increase rice straw (RS) hydrolysis. Four out of seven of the bacterial strains: *Bacillus paralicheniformis*, *Perlucidibaca piscinae*, *Bacillus clausii*, and *Bacillus mojavensis* enhanced saccharification compared to the untreated RS. In addition, pretreatment with *Bacillus paralicheniformis* increased biogas production by 1.5-fold, compared to the untreated RS.

In a study published in 2019, the effect of three different fungal strains, namely *Pleurotus ostreatus* (PO), *Phanerochaete chrysosporium* (PC), and *Ganoderma lucidum* (GL), was tested on the biodegradability of rice straw. Following a 5-week treatment period, methane yields were significantly enhanced, reaching 270, 296, and 339 mL g^{-1} VS for PO, GL, and PC, respectively. These results represent a 1.64 to 2.22-fold increase in methane production compared to the untreated control, highlighting the potential of fungal pretreatment to substantially improve the energy recovery from lignocellulosic residues (Kainthola et al., 2019a).

Another biological method for pretreating rice straw is the use of rumen fluid. In a 2016 study, the researchers attempted to overcome the recalcitrance of rice straw by employing rumen fluid. Rice straw was pretreated with rumen fluid at 39 °C for 120 h under anoxic conditions. The volatile solids produced varied, but the two main ones were acetic and propionic. According to the results of the study, the optimum pretreatment period was 24 h, while increases in biogas and methane yield reached 67% and 83%, respectively. Moreover, there was a significant reduction in the technical digestion time by 40%. Maximum methane yield reached 285 ml g^{-1} VS after a 24 h pretreatment period with rumen fluid (Zhang et al., 2016).

4.2 Physical pretreatment methods

Physical (mechanical) pretreatments aim to reduce the size of the biomass, increase the accessible surface area, reduce the degree of cellulose crystallinity, and decrease the degree of cellulose polymerization for improved digestibility (Mujtaba et al., 2023).

For instance, steam explosion involves the penetration of steam into the biomass structure, under high temperatures and pressure, followed by rapid decompression that causes mechanical breakdown of cell walls and lignin-carbohydrate bonds. This process effectively increases the accessible surface area for enzymatic accessibility while partially solubilizing hemicellulose components, substantially enhancing the methane yield potential of lignocellulosic substrates (Ziegler-Devin et al., 2021).

In the study of Zhou et al. (2016), rice straw was pretreated via steam explosion at various temperatures, ranging from 200 to 220 °C, and for two time intervals (60 to 240 seconds). Steam pretreatment significantly improved biogas production while reducing the startup period. The optimum combination (200 °C for 120 s) achieved a maximum biogas yield of 329 mL g^{-1} TS, and resulted in a 51% increase in biogas production compared to the untreated rice straw. Under these conditions, cellulose and hemicellulose degradation rates reached 54% and 50%, respectively, exceeding the control by 14% and 17%, respectively.

Extrusion pretreatment is used to effectively reduce the biomass particle size and increase its specific surface area, shortening digestion time and enhancing cellulose and hemicellulose degradation. Chen et al. (2014) evaluated the anaerobic digestion performance of extrusion-pretreated rice straw (EPRS), milling-pretreated rice straw (MPRS), and untreated rice straw (UPRS) under varying inoculum-to-substrate ratios and solid loading rates to optimize methane production. EPRS demonstrated superior performance, achieving the highest specific methane production (227 L g^{-1} VS), demonstrating an increase of 33% and 72%, compared to MPRS and UPR, respectively. Additionally, the volumetric methane production (VMP) of EPRS was 1.5 times higher compared to MPRS (Chen et al., 2014).

Extrusion pretreatment outperforms other mechanical pretreatment methods, such as chipping, grinding, and milling, because it provides continuous thermo-mechanical pressure. As a result, rice straw fibril structure expands, resulting in lower bulk density, higher water-holding capacity, and improved biodegradability. Moreover, extruders offer commercial advantages, including scalability, high shear force, and efficient mixing for large-scale applications (Gu et al., 2015).

Hydrothermal pretreatment is similar to steam pretreatment. Their fundamental difference is that steam pretreatment uses high pressure, caused by the steam, and rapid depressurization to produce mechanically shearing forces that disrupt rice straw fibers. Hydrothermal relies on hot water, under high pressure and temperature, to act as a solvent and hydrolyzing agent, dissolving the cellulose, hemicellulose, and lignin matrix (Scherzinger and Kaltschmitt, 2021).

Hydrothermal pretreatment offers substantial advantages with 40-65% higher conversion rates compared to untreated substrates and 30-50% greater glucose yields than dilute-acid pretreatment. Moreover, it is an environmentally friendly technology that significantly reduces carbon emissions and costs compared to acid pretreatment. Crucially, it reduces the corrosion rate of the reactor, extending its operational life expectancy. However, there are important issues that limit the use of hydrothermal pretreatment, such as inefficient energy utilization (62%) and the formation of inhibitory phenolic compounds (< 1.2 g/L). (Zhang et al., 2025).

Wang et al. (2018) pretreated rice straw using a hydrothermal method at various temperatures. According to their findings, pretreatment of rice biomass at 90 and 180 °C produced 307 mL g^{-1} TS biogas, while higher temperatures inhibited the production of methane. Their study indicated that soluble fractions controlled the digestion performance, while a Pearson correlation analysis showed that there is a negative relationship between methane yield and soluble fractions.

Kainthola et al. (2019b) examined the effect of electrohydrolysis (the application of direct electrical current) on rice straw biodegradability. The electrohydrolysis process utilized graphite electrodes operating at a voltage ranging from 10 to 30 V for 15-80 minutes.

Results demonstrated that electrohydrolysis pretreatment at 25 V for 60 minutes achieved maximum solubilization, equal to 55%. Moreover, batch experiments were conducted to assess the enhancement in biomethane production from improved solubilization. The experiments were conducted in 1 L batch reactors. Electrohydrolysis pretreatment at 20 V for 60 minutes effectively enhanced rice straw degradation during anaerobic digestion. Biochemical methane potential assays confirmed that CH_4 production from pretreated rice straw performed significantly better compared to the control, presenting a 42% increase (Kainthola et al., 2019b).

4.3 Chemical pretreatment methods

4.3.1 Acid pretreatment

Acid pretreatment can be conducted either with concentrated acids at low temperatures (e.g., 30-70% and 40 °C, respectively) or with dilute acids at high temperatures (e.g., 0.1% and 230 °C, respectively). Both inorganic and organic acids, including sulfuric acid (H_2SO_4), hydrochloric acid (HCl), nitric acid (HNO_3), phosphoric acid (H_3PO_4), acetic acid, and maleic acid, have been used to pretreat lignocellulosic biomass, with H_2SO_4 being the most commonly used (Baruah et al., 2018).

However, acid pretreatment presents several challenges. Specifically, the use of H_2SO4 or HNO_3 can lead to the formation of H_2S and N_2, due to the reduction of sulphate and nitrate, both of which are toxic and have the capacity to impair the biomethanation process. Moreover, pretreatment of lignocellulosic biomass at high temperatures with acid leads to the formation of hydroxymethyl furfural (HMF) and furfural, which act as strong inhibition factors during anaerobic digestion (Amin et al., 2017).

Hsu et al. (2010) investigated the effect of dilute acid on rice straw biodegradability. According to their results, rice straw pretreated with 1% (w/w) sulfuric acid (H_2SO_4) at 160 or 180 °C, for 1-5 min, resulted in significantly higher biogas production, compared to the untreated control.

Although pretreatment with acid has been tested with positive results, there are certain limitations. Specifically, Yoswathana (2010) pretreated rice straw using sulfuric acid (H_2SO_4) and an alkali at concentrations ranging from 1-9% at 121 °C. Results showed that acid-pretreated rice straw exhibited lower methane yields compared to the rice straw pretreated with the alkali or the control, under the same conditions. Lignocellulose decomposition was higher in the acid pretreatments; however, the production of inhibitory substances (furfurals) lowered the methane production compared to the alkali-pretreated and untreated rice straw.

Kocher and Kalra (2011) pretreated rice straw by soaking it in 2% H_2SO_4 (1:8) followed by autoclaving at 15 psi for 90 min. As a result, the solubility of cellulose increases by 25%, while they also observed a considerable decrease in hemicellulose and lignin contents. Pretreatment also released 70.5 mg xyloses and 296.2 mg hexoses per gram of straw in the acidic solution.

4.3.2 Alkaline pretreatment

Alkaline pretreatment uses bases, such as NaOH, $Ca(OH)_2$, KOH, and NH_4OH, to remove lignin, hemicellulose, and cellulose, rendering lignocellulosic biomass more accessible to microbes and enzymes. The effectiveness of alkaline pretreatment lies in its ability to disrupt the lignin-carbohydrate matrix that protects cellulose and hemicellulose from enzymatic hydrolysis (Bochmann, 2019; Shah et al., 2023).

The function of alkaline pretreatment is the saponification and cleavage of lignin-carbohydrate linkages. Removal of crosslinks leads to an increase in porosity and internal surface area, structural swelling, a decrease in the degree of polymerization and crystallinity, disruption of lignin structure, and a breakdown of links between lignin and other polymers (Fan et al., 2024).

Sodium hydroxide represents the most extensively studied alkaline agent for rice straw pretreatment. Apart from enhancing rice straw and other recalcitrant biomass degradability, NaOH can also facilitate the removal of heavy metals from rice straw. Specifically, in a study conducted by Xin et al. (2019), the researchers discovered that rice biomass pretreated with 6% (w/w) NaOH not only increased biogas and methane yields by 22% and 42%, respectively, but also increased the release of Cd, Pb, Cu, and Zn from 86.95 to 97.69%.

Shetty et al. (2017) pretreated milled rice straw (1 mm) with NaOH at ambient temperature for 180 min. According to their results, the highest biogas production was 514 L kg^{-1} VS day^{-1}. Takano and Hoshino (2018) pretreated rice straw with NaOH and used enzymes to increase the hydrolysis of the cellulose and hemicellulose. The cellulases were selected using multivariate analyses, while the optimum mixing ratio was calculated using response surface technology. As a result, the pretreated rice straw showed significant increases in fermentable sugars up to 80%.

Guan et al. (2018) investigated the use of $Ca(OH)_2$ combined with the liquid fraction of digestate (LFD) to pretreat rice straw. Their study showed that combined chemical-biological

(CaO-LFD) rice straw pretreatment increased the removal rate of the lignocellulosic matrix and enhanced the rupture of the ether and ester bonds between lignin and hemicellulose. Moreover, methane production was 274.65 mL g^{-1} VS, which was 57.56% greater compared to the untreated control. Guan et al. (2020) explored the effect of different temperatures and time intervals on rice biomass treated with $Ca(OH)_2$ and LFD. According to their study, RS treated with $Ca(OH)_2$ and LFD at 35 °C for two days yielded 308 mL g^{-1} VS of biomethane, producing 77.4% more than the control.

Another study investigated the pretreatment of rice biomass with NH_4OH (27% w/w) at room temperature and at 60 °C. After 3 days of incubation, the pretreated rice straw exhibited a significant reduction in lignin and silica. Also, it enhanced the cellulase accessibility to cellulose and increased the crystallinity index from 41 to 52% (Phitsuwan et al., 2017). A novel study investigated the individual and simultaneous effects of pretreating rice straw with microwaves and ammonia. According to their results, after 18 days of digestion, the microwave-assisted ammoniation method produced 282 mL g^{-1} VS, which was 25% higher than the control (Q. Liu et al., 2022).

KOH has also been used for the pretreatment of the recalcitrant rice biomass. Specifically, in a study conducted in 2020, the researchers compared the use of $Ca(OH)_2$ and KOH in the pretreatment of milled rice straw. They investigated the effect of different concentrations, different retention times, and ratios of inoculum to substrate for each alkali solution. Their results demonstrated that although both alkali solutions increased the biodegradation of the celluloses, hemicelluloses, and lignin, pretreatment with $Ca(OH)_2$ for 4 h at a 50% inoculum to substrate ratio, at 35 °C, increased the production of VFA by 47% (Guardia-Puebla et al., 2020).

5.1 Rice straw co-digestion with livestock waste

The high carbon-to-nitrogen ratio of rice straw, typically between 25 and 75 (Meng et al., 2018; Wei et al., 2025), and its recalcitrant nature are the primary factors limiting its efficient anaerobic digestion. The low nitrogen content of rice straw limits the growth of the anaerobic microbial community, hindering efficient digestion. Co-digestion with nitrogen-rich livestock wastes appears to be the optimum strategy to address rice straw's nitrogen deficiency and constrained nutrient availability (Ferdeș et al., 2023; Kadam et al., 2024a). Moreover, co-digestion of rice straw with livestock wastes not only increases the availability of AD important nutrients, such as nitrogen, in the feedstock but also promotes synergies due to enhanced microbial diversity.

5.1.1 Co-digestion with swine waste

Pig waste encompasses both manure and urine, each possessing distinct characteristics. The integration of nitrogen-rich and carbon-rich substrates addresses multiple limitations simultaneously, including nutrient imbalance, insufficient buffering capacity, and limited microbial diversity that typically constrain rice straw mono-digestion.

According to Zhong et al. (2021), pretreatment of rice straw with a cellulolytic microflora and co-digestion with swine manure can increase biogas production and process stability, compared to the mono-digestion of swine manure. Tian et al. (2023) demonstrated that co-digestion of pig manure with rice straw enhanced biogas production due to the increased microbial biodiversity and synergy stemming from the co-digestion process. Importantly, the system exhibited reduced ammonia formation compared to the other co-digestion scenarios, mitigating the risk of inhibition from excessive nitrogen release. This is particularly significant given that high total ammonia nitrogen concentrations, coupled with elevated pH and temperature, constitute conditions toxic to the microbial community, and especially to the methanogenic archaea (Bardi et al., 2025; Heng Wu et al., 2025).

Moreover, when rice straw was co-digested with pig manure and kitchen waste, the increased nutrient balance was the deciding factor, enhancing methane production significantly (72%) compared to mono-digestion (Ye et al., 2013). This enhancement reflects the synergistic effect of bacterial diversification and the complementary action of nutrients from multiple wastes (Amos et al., 2025; Du et al., 2019; Ganesh Saratale et al., 2018).

However, operational stability in pig manure co-digestion systems is highly sensitive to organic loading rate. Moderate loading rates maintain stable operation, while excessive organic loads cause rapid accumulation of volatile fatty acids (VFAs), leading to system acidification and potential failure (Basak et al., 2025; Duong and Lim, 2025). Rusanowska et al. (2025) reported that an OLR of 2 and 3 kg VS $(m^3d)^{-1}$ resulted in stable and high methane yields up to 446 L kg^{-1} VS. In contrast, an increased organic load of 4 kg VS $(m^3d)^{-1}$ led to severe process disruption and a pH drop from 7.7 to 6.1. Co-digestion with swine manure can also cause foaming (Daly and Ni, 2024; Fierro et al., 2016). The main causes of foaming are surface-active compounds, excessive organic loads, significant temperature variations, excessive surfactants, and the presence of microbial genera linked to foam formation (He et al., 2017; C. Jiang et al., 2021). Foaming can be mitigated through mixing and appropriate reactor design and geometry, as well as the use of antifoams and defoaming agents (Mokoena and Matambo, 2025).

Meng et al. (2018) examined the effect of feedstock-to-inoculum ratio (F/I) on biogas production and the microbial community in a co-digestion experiment involving pig urine and rice straw in a solid-state thermophilic reactor. According to their results, lower F/I ratios consistently presented higher biogas yields while facilitating the maintenance of pH and ammonia levels at appropriate levels. Importantly, they observed no VFA inhibition across the F/I tested samples, suggesting that pig urine has excellent buffering capacity. Additionally, according to Said et al. (2015), rice straw combined with high inoculum levels enhances the digester's volumetric utilization efficiency. Consequently, the volume of the rice biomass is reduced via rapid degradation, resulting in improved digester and process efficiency.

Biological augmentation through cellulolytic microbial consortia is a novel methodology employed to enhance biogas production through co-digestion with swine manure. Shen et al. (2018) demonstrated that the addition of a cellulolytic microbial consortia to pretreat rice straw and pig manure at an OLR of 2.5 kg COD $(m^3d)^{-1}$ under mesophilic conditions (35-37 °C) increased volumetric methane production rate by 62.4% and methane yield by 37.8%. The mechanism involved biological solubilization that disrupted the complex lignocellulosic matrix, releasing cellulose and hemicellulose to enhance microbial accessibility.

5.2 Co-digestion with cattle waste

Cow manure co-digestion offers important advantages in terms of enhanced production stability, nutrient availability, biomethanation, and buffering capacity (C. Jiang et al., 2021). The addition of cow manure in AD also provides trace elements, crucial for stable digestion (Muhayodin et al., 2021). Moreover, cow manure has a lower nitrogen content compared to swine manure (Sihlangu et al., 2024b) and thus, the risk of excessive ammonia formation is considerably lower. Therefore, co-digestion with cow manure enhances process stability and lowers the risk of ammonia inhibition (Kadam et al., 2024a).

However, cow manure is a nitrogen-rich substrate, and therefore, co-digestion with it can lead to excessive VFA accumulation under high OLRs. This is evident from the study of Li et al. (2015), who reported significant VFA buildup, with total concentrations reaching 12,4 mg L^{-1}, when the OLR was raised to 12 kg VS $(m^3d)^{-1}$. As a result, the pH decreased from 7.0 to 5.4, severely compromising the methanogenesis process.

Supplementation with trace elements appears to be a promising strategy to enhance cow manure co-digestion performance. Cai et al. (2017) demonstrated that Mn, Se, and Mo addition at low, medium, and high concentrations increased methane yield by up to 144%, 138%, and 141%, respectively. Muhayodin et al. (2021) reported that Co, Mo, Cu, and Zn remained stable and in adequate quantities throughout the AD process; hence, external addition is unnecessary. In contrast, Fe and Ni appeared to diminish towards the end of the process for unknown reasons. Similar to Cai et al. (2017), Muhayodin et al. (2021) suggested the supplementation with Se when co-digesting cow manure with rice straw, as this element appears to be lacking in the cow manure-rice straw feedstock. These trace elements play a key role in the production of enzymes associated with VFA degradation and synthesis. Moreover, supplementation with these elements alters the composition and diversity of the microbes participating in the anaerobic degradation process and therefore enhances the efficiency of the process (Cai et al., 2017). Additionally, several studies have noted that the digestate from the liquid AD of rice straw with cow manure is an excellent inoculum for solid-state AD systems (Ge et al., 2016; Luo et al., 2018).

5.3 Co-digestion with poultry waste

Poultry manure, particularly from chicken, exhibits the highest nitrogen content compared to all the other livestock wastes (L. Zhang et al., 2023), making it both a promising and challenging co-substrate for rice straw co-digestion. Poultry manure appears to be an ideal candidate for co-digestion with rice straw because it can optimize the C:N ratio in the feedstock mixture, as the high carbon content of rice straw can be effectively balanced by the nitrogen-rich poultry manure (Mothe and Polisetty, 2021). However, the significantly high nitrogen content of poultry manure also introduces a considerable risk of ammonia inhibition, requiring constant monitoring.

Mei et al. (2016) investigated the effects of organic loading rate on process stability and performance in rice straw-chicken manure co-digestion. The study emphasized that substrate ratio optimization is critical, as excessive poultry manure addition can lead to ammonia accumulation that inhibits methanogenic activity. Substrates, such as poultry manure with high nitrogen content, usually have an elevated total ammonia nitrogen (TAN) content. Increased TAN levels coupled with high temperatures and pH lead to increased free ammonia (NH_3) concentration over ammonium (NH_4^+), which is significantly more toxic to the methanogenic archaea (Cai et al., 2021).

Babaee et al. (2013) examined the effects of different organic loading rates and temperatures on the biogas and methane production of anaerobically co-digested poultry manure and straw. The study highlighted that co-digestion at mesophilic temperatures enhances methane and biogas production. The use of different substrates enhanced the diversification of the microbial population and offered greater nutrient diversity and availability. Specifically, poultry manure inherently contains a diverse and indigenous group of cellulolytic bacteria that could facilitate increased degradation of recalcitrant organic materials such as rice straw (Zainudin et al., 2022). Therefore, co-digestion of rice straw with poultry manure exhibited significant synergistic actions that are invaluable in enhancing the hydrolysis of rice straw and the overall anaerobic degradation process (Zainudin et al., 2022).

Beyond nutrient balancing, poultry waste co-digestion addresses multiple operational challenges, including inconsistent feedstock characteristics, acidification tendencies, trace element deficiencies, and suboptimal buffering capacity (Zhang et al., 2018).

6.1 Digestate as an organic fertilizer

Land mismanagement and climate change in the Mediterranean region, in conjunction with the consumers' needs for organically sourced food, necessitate the adoption of alternative agricultural systems. Conventional farming practices need to be replaced with novel methods that embrace the concepts of circular economy and nutrient recirculation.

The watery residue remaining after the end of the AD process is a valuable by-product containing nutrients necessary for plant development, called digestate (Kabeyi and Olanrewaju, 2022).

Digestate has the capacity to provide essential nutrients to the plants, while simultaneously enhancing soil health and mechanical properties (Hasan et al., 2025). Furthermore, digestate has beneficial microorganisms that can accelerate organic matter decomposition in the soil, mobilize insoluble phosphorus, and increase nutrient availability (Zapata-Morales and Moreno-Andrade, 2025).

Anaerobic digestion fundamentally restructures the chemical composition and biological characteristics of the original feedstock (Alburquerque et al., 2012). Therefore, digestate exhibits diverse features, such as partially stabilized organic matter, concentrated nutrient fractions, and microbial communities adapted to anaerobic environments.

The use of digestate is not only a highly effective organic fertilizer and soil amendment, but simultaneously reduces the odors from odorous organic wastes. Furthermore, it facilitates climate change mitigation through carbon sequestration in soils and the capture of methane during the AD process (Martín-Sanz-Garrido et al., 2025a). Developing effective organic fertilizers has become increasingly important for future agriculture, with various biofertilizer types, including animal manures, composts, and vegetable byproducts, showing successful application across various agricultural settings (Folina et al., 2025; Khan et al., 2024; Khanyile et al., 2024; Rahman et al., 2025).

Conventionally cultivated cereal crops such as rice and maize require substantial fertilizer and pesticide inputs (Choudhary et al., 2023). However, excessive fertilization, particularly nitrogen, can extend the crop's biological cycle, delaying maturity while simultaneously increasing susceptibility to pathogenic organisms (Ahmed et al., 2017; Marcianò et al., 2023; Y. Sun et al., 2020). Comparative analyses of various organic wastes used as fertilizers, including food waste, sewage sludge, and biogas production residues, have revealed that biogas-derived digestate contains the highest concentrations of organic matter, total carbon, and nitrogen (Višniauskė et al., 2018). Moreover, field trials have demonstrated that fertilizing spring barley with composts derived from food waste and biogas production waste resulted in significant yield increases (Višniauskė et al., 2018). Therefore, digestate appears as an ideal candidate for use as organic fertilizer in sustainable and organic agricultural systems.

6.1.1 Essential plant nutrients in digestate

Digestate provides a broad spectrum of essential nutrients, including nitrogen, phosphorus, and potassium, that are crucial for plant growth and development (Chojnacka and Moustakas, 2024; Zapata-Morales and Moreno-Andrade, 2025).

Nitrogen in digestate exists predominantly in ammonium form, readily available for plant use. This characteristic has been demonstrated in citrus cultivation trials, where digestate application increased chlorophyll content by approximately 50% compared to conventional mineral fertilizers (Torrisi et al., 2021). Although nitrate nitrogen is the preferred form for immediate absorption by the plants, ammonium nitrogen is also used by the plants, albeit in smaller quantities. Nevertheless, some plants prefer ammonium nitrogen over nitrate nitrogen, such as rice, while ammonium nitrogen is less mobile and therefore more difficult to leach into the soil (Mian et al., 2009).

Hammerschmiedt et al. (2022) reported that digestate, especially derived from multiple substrates, can increase soil phosphorus availability, while García-López et al. (2023) noted that adding digestate to the soil alters the biogeochemistry of soil phosphorus and has the capacity to elevate both organic and inorganic phosphorus soil fractions. As a result, the availability of phosphorus in the soil for plant use increases, leading to the enhancement of plant traits associated with phosphorous fertilization, such as a healthy and long root system (Khan et al., 2023; Ma et al., 2020).

Moreover, Okoli et al. (2023) demonstrated that digestate increases soil available K content, while Yuan et al. (2023) showed that the combined application of inorganic and organic fertilizers leads to enhanced K uptake from sweet potato plants.

Hammerschmiedt et al. (2022) and Rolka et al. (2024) both reported increases in micronutrient availability in the soil post-digestate application, enhancing crop yield. All of the aforementioned studies point to the fact that digestate not only possesses valuable nutrients essential for plant nutrition, but more importantly, these nutrients exist in forms readily available for plant use.

6.2 Nutrient distribution between fractions

Separating digestate into two fractions (liquid and solid) is almost universally practiced (Dincă et al., 2025). The liquid fraction is rich in soluble fast-releasing nutrients, such as soluble nitrogen, and is suitable for fertigation systems providing rapid nutrient availability. In contrast, the solid fraction, which mostly contains stable organic matter and phosphorus, enhances soil structure and provides nutrients at a slower rate (Jankauskienė et al., 2024).

Digestate composition relies on the substrates used for its production, process engineering parameters, and digestion handling procedures; therefore, it is almost impossible for two digestates to have the same composition (Czekała et al., 2022). Depending on the source material, digestates can possess different qualities and hence can be utilized for different plants and cropping systems.

Digestate separation into liquid and solid form provides the additional advantage of obtaining two fractions with distinct characteristics. Recent investigations have examined how dry matter, nitrogen, phosphorus, and heavy metals distribute between solid and liquid phases. Research has revealed that the solid fraction predominantly contains dry matter, phosphorus, and organic carbon, whereas the liquid fraction primarily has nitrogen in ammonium form (Egene et al., 2021).

Phosphorus predominantly concentrates in the solid phase, where phosphate ions are released from the organic matter (Bolzonella et al., 2018). Conversely, most organic nitrogen and potassium reside in the liquid phase of digestate (Slepetiene et al., 2023). These findings indicate that digestate fractionation can serve as an effective method for extracting valuable nutrients while potentially reducing environmental contamination. Moreover, some researchers have indicated that solid digestate shows potential for use as growing media and/or fertilizer in greenhouse vegetable production (Bergstrand et al., 2020; Stoknes et al., 2018, 2016).

Beyond macronutrients, digestate supplies calcium, magnesium, sulfur, and trace elements, including iron, manganese, zinc, and copper. Calcium concentrations can reach 47.6 g kg^{-1} in digestates from certain feedstocks, providing this essential element for cell wall structure and cellular signaling processes (Zapata-Morales and Moreno-Andrade, 2025). The biological significance of these secondary nutrients is crucial for some of the most important plant functions. Specifically, magnesium forms the central component of chlorophyll molecules, iron participates in electron transport chains, and zinc activates numerous plant enzymes.

6.3 Nutrient release characteristics

Conventional fertilizers typically provide nutrients in soluble forms, causing an initial spike in soil nutrients followed by a rapid decline owing to crop uptake, leaching, or nutrient volatilization. In contrast, nitrogen in digestate is mostly in ammonium and organic form. Ammonium nitrogen has to go through a nitrification process to be easily absorbed by most plants, which reduces leaching, while the organic nitrogen fraction mineralizes gradually, extending nutrient supply over weeks or months (Möller and Müller, 2012; Santos et al., 2025).

The slow-release capabilities of digestate have significant advantages and disadvantages. Slow-release fertilizers like digestate provide a sustained stream of nutrients and reduce nutrient leaching (Valentinuzzi et al., 2020). However, slow release means that it is difficult to precisely predict the amount of nutrients released per unit of time, as the rate of mineralization can vary depending on environmental conditions such as temperature, moisture, and soil biological activity.

6.4 Digestate treatment

Lowering digestate pH through acid addition can improve nutrient bioavailability, reduce ammonia volatilization, and decrease pathogen viability, in a post-treatment process known as acidification. Sica and Magid (2024), in a seed germination experiment, demonstrated that acidification with H_2SO_4 to pH 6.5 enhances seed germination rates and reduces the need for digestate dilution.

Acidification combined with solar drying is another post-treatment strategy. Adding acids to digestate reduces pH, converts ammonia to ammonium, reduces volatilization losses, and reduces GHG emissions. Acidified digestate can significantly increase seed germination, likely due to reduced ammonia toxicity. Solar treatment is used as a low-cost treatment strategy to reduce the water content of digestate (Morey et al., 2023). However, acidification requires careful pH control, which increases the post-treatment cost.

Biofiltration and vermifiltration are post-treatment techniques able to reduce suspended solids, contaminants, and heavy metal concentrations. Ziegler-Rodriguez et al. (2023) compared sand biofiltration and vermifiltration for treating digestates from cattle manure and whey.

Vermifiltration, using worms to decompose organic matter, effectively reduced heavy metal contamination and resulted in a lower carbon footprint.

Technologies such as membrane filtration, evaporation, and struvite precipitation can concentrate nutrients from liquid digestate, producing more concentrated fertilizer products while reducing transport costs and application volumes (Zamanzadeh et al., 2016).

Aerobic composting of solid digestate fractions further stabilizes organic matter, reduces moisture content, facilitates pathogen elimination, and improves handling characteristics (Czekała et al., 2020). However, composting incurs additional costs and may result in some nutrient losses through volatilization.

Moreover, digestate composting via certain methods, such as C:N adjustment and the use of microbial and chemical amendments, can reduce nitrogen losses through ammonia volatilization. Additionally, nitrogen losses before composting can reach 60-70%, while a stable digestate compost has the capacity to experience markedly lower nitrogen losses (Manu et al., 2021).

6.5 Use of digestate in hydroponic systems

Growing vegetables using hydroponic systems offers several advantages compared to soil-based cultivation, including increased water and nutrient utilization efficiency, enhanced productivity per unit area, decreased susceptibility to plant pests, and independence from soil constraints (Putra and Yuliando, 2015). Research on using digestate as a nutrient source for hydroponic crop production has been reported by multiple researchers (Krishnasamy et al., 2012; Sela Saldinger et al., 2023).

Studies on using digestate for hydroponic vegetable production have yielded contradictory results. Research has shown that dissolved organic matter from digestate exhibits auxin-like effects on plants (Scaglia et al., 2015). However, some studies suggest digestate may prove unsuitable for soilless greenhouse horticulture due to potential toxicity (Mupambwa et al., 2019) and bacterial contamination (Södergren et al., 2022).

The risk of ammonia phytotoxicity due to elevated levels of ammonia in digestate necessitates the conversion of ammonium nitrogen to nitrate nitrogen prior to implementation as a hydroponic fertilizer (Mupambwa et al., 2019). The nitrification process can be easily performed by nitrification bacteria upon digestate aeration. However, there is no clear methodology for controlling and monitoring digestate nitrification in hydroponic systems (van Rooyen and Nicol, 2022).

Research has focused on reducing ammonium concentrations. One such method utilizes moving bed biofilm reactors, a technology commonly employed in water treatment applications, to reduce ammonium via biological nitrification in aerobic conditions (Jang et al., 2023). Moreover, research revealed that using digestate as a liquid fertilizer can significantly reduce the cultivation period of bok choy compared to conventional mineral fertilizer, up to 20% (Pelayo Lind et al., 2021).

6.6 Feedstock sources and compositional variation

Digestate is produced from various organic sources, including food and municipal waste, energy crops such as maize and grass silage, and byproducts from the food and agro-industrial sector, which can include animal byproducts and spent grains from brewing. Therefore, the specific feedstocks used in digestate production can influence the nutrient content and properties of the resulting digestate (Wystalska and Kwarciak-Kozłowska, 2023).

Digestate physicochemical properties exhibit substantial variability depending on substrate type and composition, as well as operational parameters of the anaerobic digestion process. Generally, digestate has lower total solids, total organic carbon (TOC), and viscosity than its source materials, along with a reduced carbon-to-nitrogen (C:N) ratio (Jankauskienė et al., 2024). The anaerobic digestion process breaks down organic matter, which lowers the concentration of the aforementioned traits (Jankauskienė et al., 2024). Conversely, pH and ammonium (NH_4^+) concentrations typically increase in digestate due to the anaerobic digestion process. Anaerobic digestion converts urea and organic nitrogen into ammonium, a process that can lead to an increase in pH, which shifts the equilibrium towards free ammonia (NH_3) generation rather than ammonium (NH_4^+), which is highly toxic to the methanogenic archaea (Sigdel et al., 2025).

Digestates derived from protein-rich substrates such as slaughterhouse waste, food scraps, or manure naturally contain elevated nitrogen concentrations, sometimes reaching 5% on a dry weight basis (Chojnacka and Moustakas, 2024). Meanwhile, digestates from agricultural residues or municipal green waste typically show lower nitrogen concentrations but higher carbon content. Hence, depending on the mixture, the physicochemical and nutrient composition of each digestate can vary significantly (van Midden et al., 2023).

Chong et al. (2022) performed a comprehensive nutritional analysis of the different digestates, revealing great variation depending on the material sourced for their production. According to their results, sewage sludge digestates contain phosphorus ranging from 6 to 30 g kg^{-1}, potassium from 1 to 10 g kg^{-1}, sulfur from 11 to 17 g kg^{-1}, and iron from 24 to 79 g kg^{-1}, with pH values spanning 5.8 to 13.7 (Hossain et al., 2021; Niemiec et al., 2022; Xu et al., 2021). Livestock waste digestates demonstrate high phosphorus content (16.8 g kg^{-1} for poultry), substantial potassium levels (21.2 g kg^{-1} for poultry), and notably high iron content (1497.5 g kg^{-1} for poultry), with pH values ranging between 6.6 and 7.9 (Hossain et al., 2021; Niemiec et al., 2022; Xu et al., 2021). Energy crops show moderate phosphorus content (2.495 g kg^{-1} for maize) and potassium (12.3 g kg^{-1} for maize) levels, with pH values around 10-10.6. The carbon-to-nitrogen ratio proves notably higher at ~30 for maize silage (Arif et al., 2018; Škarpa et al., 2021; Xu et al., 2021).

Nitrogen concentration in digestates shows an even greater variability, with Kjeldahl nitrogen values ranging from 0.4 to 2 g kg^{-1} in agricultural wastes and from 18 to 53 g kg^{-1} in sewage sludges and animal wastes (Katakula et al., 2021; Najafi et al., 2023; Rahman et al., 2022).

6.7 Economic aspects and market development

Europe's total annual digestate production is estimated at 56 million tons of fresh matter per year (EBA Statistical Report, 2024). Market pricing for digestate varies considerably based on processing method and quality characteristics. Dried digestate prices range from 5 to 30 € per ton, depending on feedstock source, nutrient content, and quality characteristics. Wet digestate is typically sold for 0 to 8 € per ton, while composted digestate generally ranges between 0 and 50 € per ton (EBA Statistical Report, 2024).

Additionally, Jurgutis et al. (2021) reported that the approximate daily value of digestate derived from a 1 MW biogas power plant is 1,518 €. Exploiting digestate sales would markedly increase the profitability of the biogas plants if it were a widely marketable product.

7.1 Effects of digestate on soil and plant growth

7.1.1 Soil chemical properties

Application of digestate influences several soil chemical properties related to nutrient cycling. Studies have demonstrated that digestate incorporation can enhance soil cation exchange capacity (CEC), improving the soil's ability to retain and supply essential cations, including calcium (Ca), magnesium (Mg), potassium (K), and ammonium (NH_4) (Elumalai et al., 2025). This enhancement of CEC contributes to improved nutrient retention and reduced leaching losses.

The carbon content of digestates, which ranges from 13 to 42% depending on the substrate and process conditions (Fernández-Bayo et al., 2017; Peng and Pivato, 2019a), is a source of organic matter able to improve soil structure and water-holding capacity. Moreover, several studies have noted that digestate organic matter mineralization leads to the gradual release of trace elements, increasing their mobility and bioavailability (Audry et al., 2011; Ding et al., 2025).

7.2 Soil physical properties

Organic matter in digestate has the potential to enhance soil physical characteristics. Digestate addition has been reported to enhance soil aeration and water retention capacity without adversely affecting soil structure when applied at appropriate rates (Gryta et al., 2025). Solid digestate has a higher fiber content and, therefore, is able to substantially improve soil physical properties compared to liquid digestate (van Midden et al., 2023).

Moreover, Odlare et al. (2008) demonstrated that compared to other organic amendments, including pig manure, cow manure, and compost, soils treated with liquid digestate from municipal waste exhibited sustained improvements in physical and biological properties over a four-year period. Thereby, exhibiting digestates ability to enhance soil physicochemical properties when applied continuously for years.

7.3 Microbial effects

7.3.1 Soil microbial biomass and activity

Digestate also has the capacity to stimulate soil microbial biomass and activity. Digestate contains partially degraded organic matter, microbial biomass, and inorganic compounds that serve as substrates for soil microorganisms (Senevirathne and Kaparaju, 2025a). Application of digestate has been consistently associated with increases in microbial enzyme activities, particularly dehydrogenase, which serves as an indicator of overall soil biological health (Doyeni et al., 2023).

However, the effect of digestate addition on microbial health, growth, and diversification can vary depending on the fraction applied. The application of liquid digestate results in rapid microbial activity, followed by an equally rapid reduction in microbial activity within days or weeks post-application (Barduca et al., 2021). The short-term effect of liquid digestate addition can be traced to the insufficient carbon content of the liquid fraction, leading to unsustainable long-term microbial growth (Iocoli et al., 2019). In contrast, solid digestate applications lead to more sustained increases in microbial biomass and activity, as solid digestate has a higher carbon content (Badagliacca et al., 2020; Cattin et al., 2021).

Almeida et al. (2019) reported a substantial increase in microbial activity leading to enhanced organic matter mineralization following digestate application. Other researchers have reported that the digestate's solid fraction has a positive impact on all groups of soil microorganisms. Nevertheless, the liquid fraction can benefit bacteria but could negatively affect mycorrhizal and saprophytic fungi (van Midden et al., 2023).

7.3.2 Beneficial microorganisms and plant growth promotion

Microbiological analyses of digestates have consistently revealed the presence of beneficial bacteria with plant growth-promoting capabilities. Dominant phyla include Firmicutes and Proteobacteria, with particularly high abundances of *Pseudomonas* and *Bacillus* genera (Fernandes et al., 2020a; Pagliaccia et al., 2020; Song et al., 2021). These bacterial groups promote plant growth in numerous ways, including phosphate solubilization, siderophore production for iron acquisition, and synthesis of bioactive compounds (Fatima et al., 2024; Majeed et al., 2015; Ren et al., 2020).

Pagliaccia et al. (2020) identified specific strains with particularly strong plant growth-promoting characteristics, including *Pseudomonas* spp., *Acinetobacter* spp., *Glutamicibacter soli*, and several other species. Bioinformatics analyses revealed that these bacteria possess genes associated with siderophore production, iron acquisition systems, phosphate solubilization, and nutrient facilitation. Moreover, when digestate was applied to citrus nurseries via drip irrigation, soil nitrogen increased by 152-166% and carbon by 164-259%, while the population of these beneficial bacteria increased substantially (Pagliaccia et al., 2020).

Digestates produced from specific substrates can be enriched for particular functional groups. Caballero et al. (2020) demonstrated that digestates from pasteurized buttermilk enhanced the growth of lactic acid-producing bacteria (*Lactobacillus rhamnosus*), which are capable of biocontrol activity against phytopathogenic microorganisms. This represents a more targeted approach toward producing agricultural probiotics with specific rhizosphere functions.

7.3.3 Prebiotic effects

Digestates not only introduce beneficial microorganisms to the soil but also can effectively function as prebiotics. Specifically, digestates contain considerable amounts of residual organic carbon, humic acids, fulvic acids, carboxylic acids, amino acids, fatty acids, and other bioactive compounds that can stimulate plant growth and microbial activity (O'Connor et al., 2022; Scaglia et al., 2015).

The C:N ratio of digestates also influences the microbial communities of the soil. Fernández-Bayo et al. (2017) demonstrated that lower C:N ratios (below 23) increased the expression of microbial genes related to the degradation of hemicellulose and lignin. Conversely, higher C:N ratios (above 27) enriched the Clostridiales family, suggesting that adjusting the C:N ratio can steer the microbial community towards targeted microbial traits and populations.

García-Sánchez et al. (2015) evaluated digestate application at field-equivalent rates (100 t ha^{-1}) and observed significant increases in dehydrogenase activity at 60 days, indicating enhanced biological activity. The relative abundance of the 16S rRNA gene increased at all treatment intervals, with the highest increases recorded at 60 days post-digestate implementation, suggesting that digestate stimulates soil microbial diversity and abundance. However, certain enzyme activities, such as *β*-glucosidase, remained unchanged, indicating selective effects on different aspects of soil biological functioning.

The dissolved organic carbon (DOC) concentration in digestates also plays a critical role in determining microbial responses. DOC concentrations exceeding typical field doses led to enhanced activity of soil microbes and population size for multiple weeks (Manfredini et al., 2021; Fernandes et al., 2020).

7.4 Germination and early growth

Digestate application can significantly enhance or suppress seed germination depending on digestate dilution, additives, crop species, and digestate characteristics. Some studies claim that digestates, especially those possessing high salinity, can seriously hinder seed germination, while others reported that properly diluted or supplemented digestates can significantly enhance seed germination. Specifically, Twongyere (2025) reported that digestate with high electrical conductivity can seriously undermine seed germination, while supplementation with biochar led to significant seed germination.

Processing digestate prior to application can significantly mitigate any potential toxicity issues and enhance its nutrient availability. Specifically, Fernandes et al. (2020b) treated digestate with membrane filtration, settlement, and dilution (2.5%) and used it to grow macroalgae. According to their results, the treated digestate exhibited increased soluble phosphorus and nitrogen availability, and appeared to be a promising fertilizer for *Chlorella vulgaris* cultivation. Moreover, Castro-Rivera et al. (2020) demonstrated that digestate from bovine manure with tomato crop residues stimulated root elongation in lettuce without causing phytotoxicity, suggesting potential use as a germination enhancer. However, excessive digestate concentrations can inhibit germination. High concentrations of ammonium and especially free ammonia in digestate are toxic for plants and microalgae, while the high salt content also compromises plant and microalgae development (Esteban et al., 2016; R. Jiang et al., 2021; Kovačić et al., 2022a).

Cichy et al. (2024) showed that digestate from food waste, combined with fish sludge, significantly impaired seed germination and plant growth of garden cress and wheat plants. The researchers noted that digestates with high TOC, DOC, and TOC/TN ratio are not suitable for plant growth. In contrast, pre-incubating the digestate in the soil prior to use can sufficiently reduce TOC, DOC, and TOC/TN ratio, rendering digestate an efficient biofertilizer. The researchers observed that after a short period, the inhibitory effects of digestate were drastically reduced, consistent with other studies (Möller and Müller, 2012), highlighting that proper digestate maturity renders it an efficient organic fertilizer.

7.5 Vegetative growth and biomass production

Digestion application to plants post-germination usually promotes vigorous vegetative growth and enhanced biomass accumulation. Mickan et al. (2022) noted that using digestate as fertilizer for tomato plants resulted in improved shoot and root growth and thus overall crop biomass. Łukowiak et al. (2025) reported that winter oilseed rape (WOSR) plants fertilized with digestate demonstrated higher yields, especially those receiving a combined mineral-digestate fertilization. Moreover, the increase in yield was significantly correlated with the digestate's Mg content, which regulated Ca uptake. Digestate's ability to reduce the Ca uptake from the WOSR plants led to enhanced nitrogen and Zn uptake and thus to higher yields.

Some studies have reported that they have identified the optimum digestate application rates, resulting in maximizing growth benefits while avoiding inhibitory effects. Coaguila et al. (2019) assessed the effect of untreated digestate from cow and pig manure containing moderate levels of potassium (810 mg L^{-1}), phosphorus (73 mg L^{-1}), and nitrogen (5 mg L^{-1}) in onion plants. According to their results, a 50% dilution of digestate resulted in enhanced plant height and biomass. Therefore, it appears that even raw digestate, when efficiently diluted, can be directly used effectively.

Vaish et al. (2022) investigated the effect of digestate on the physicochemical characteristics of the soil, growth parameters, and the yield of aubergine (*Solanum melongena* L.), in a pot experiment. According to their results, digestate fertilization increased total organic carbon (TOC), exchangeable Na, K, Ca, P, and total Kjeldahl nitrogen (TKN) in the soil. In addition, total chlorophyll, carotenoid, maximum protein content, and yield were also significantly increased in the aubergine plants. Some adverse effects were also reported, such as increased metal content and increases in proline, peroxidase, and lipid peroxidation. However, metals remained within safe limits, and increases in ascorbic phenols (up to 24%) alleviated any potential issues from oxidative stress.

Digestate combined with biochar can serve as an excellent alternative fertilizer, increasing yields and soil quality, while reducing greenhouse gas emissions. Zheng et al. (2025) reported that digestate coupled with biochar produced from the pyrolysis of eucalyptus trimmings proved ideal for the cultivation of rye, even though the concentration of mineral nitrogen in digestate was lower compared to the control (rye plants fertilized with urea). Additionally, greenhouse gas emissions were significantly reduced, and soil biological health increased 1.4-fold.

Digestate has also been successfully employed in the cultivation of faba beans, sorghum, and tomato plants. Particularly, Alhammad and Seleiman (2023) observed enhanced plant height, leaf area, dry leaf weight, stem dry weight, number of branches, number of seeds, 100-seed weight, and yield of faba beans when using a combination of 50% digestate and 50% biofertilizer. Digestate enriched with macronutrients also led to significant increases in the yields of sorghum and maize (Kordas et al., 2024). The yield of tomato plants was also increased when digestate was used in combination with soil amendments, with increases reaching 17.3% (Tiong et al., 2024).

7.6 Stress tolerance and disease resistance

The microbial communities introduced or stimulated by digestate application may also contribute to disease suppression. Laboratory experiments have demonstrated significant reductions in root-knot nematode populations and decreased egg production by cyst nematodes in digestate-treated soils compared to untreated soils (Das et al., 2022; Xiao et al., 2007). These nematode-suppressive effects are attributed to multiple mechanisms, including stimulation of antagonistic bacterial communities, presence of plant-derived nematocidal compounds in digestate mixtures, and elevated concentrations of ammonium and organic acids generated during anaerobic digestion (Min et al., 2007; Wang et al., 2019; Westphal et al., 2016).

Oldani et al. (2023) examined digestate's nematocidal activity against the plant-parasitic nematode *Meloidogyne incognita*. According to their results, in vitro application of 10% diluted digestate resulted in more than 60% mortality of the second-stage juveniles after 24 h, while after 7 days the mortality rate exceeded 90%. Moreover, the addition of 10% and 5% diluted digestate led to the prevention of root galling in two highly susceptible vegetable plants (cucumber and tomato).

7.7 Bioactive compounds and growth promotion

Digestates contain various bioactive compounds that can directly stimulate plant growth beyond their nutritional value. These include humic acids, fulvic acids, amino acids, fatty acids, auxins, gibberellins, and other plant hormones (Scaglia et al., 2015). The presence and concentration of these compounds depend on the original feedstock and the anaerobic digestion conditions.

Volatile fatty acids (VFAs), particularly acetic acid, represent one class of metabolites found in digestates that have been shown to have a positive impact on soil and plant health. Acetic acid typically accounts for approximately 34% of digestate composition, followed by propionic acid at 5.8% (Ghidotti et al., 2018; Oldani et al., 2023). Utsumi et al. (2019) reported enhanced drought resistance in cassava plants treated externally with acetic acid by increasing ABA levels and the expression of drought-resistant related genes. Although these compounds can exhibit antimicrobial properties against soil pathogens at appropriate concentrations, their effects on plants require careful application to avoid negative impacts (Montana State University, 2021).

Samaniego-Gaxiola and Balagurusamy (2013) demonstrated that VFAs such as acetic, butyric, formic, and propionic acids at concentrations of 3-6 μg L^{-1} inhibited *Phymatotrichopsis omnivore*, a soil-borne ascomycete that causes "Texas rot" disease affecting thousands of plant species. Similarly, several studies have reported apoptosis and necrosis of pathogenic fungi cells, such as *Candida albicans* and *Saccharomyces cerevisiae*, with the use of acetic acid in concentrations exceeding 200 millimolar (Ludovico et al., 2001; Ramsdale, 2008). These findings emphasize that digestates provide not only nutrients but also compounds that can support crop health by inhibiting disease-causing microorganisms.

8.1 Use of Digestate as fertilizer for aromatic and medicinal plants

Medicinal and aromatic plants (MAPs) constitute key botanical resources, providing indispensable raw materials for the flavor, cosmetic, and pharmaceutical industries (Zamani et al., 2025). The utilization of digestate in MAP cultivation offers a sustainable solution for MAP production. Furthermore, because peat, the predominant growing medium in EU greenhouse production (Taparia et al., 2021), poses serious environmental drawbacks (Gruda et al., 2024), using solid digestate as a potential peat substitute, particularly in pot production, significantly increases the value of digestate (Asp et al., 2022).

8.1.1. Biomass yield and growth parameters

Several studies have reported the effective use of solid digestate (SD) as a peat substitute in MAP pot systems (Asp et al., 2022; Dubský et al., 2019; Greco et al., 2021). Specifically, Asp et al. (2022) demonstrated that substituting peat with SD at a 50% ratio resulted in yields equivalent to those achieved when using conventionally fertilized peat alone. Greco et al. (2021) reported that digestate can partially substitute peat in the soilless cultivation of sage. Therefore, digestate appears to be an efficient partial growing substrate, while providing nutrients for plant development and thus decreasing the need for additional mineral fertilization without compromising crop yield. However, caution is imperative, as high digestate concentrations, such as > 80%, have been observed to suppress MAPs growth (Asp et al., 2022).

The efficacy of digestate as a nutrient source is also highlighted in the cultivation and development of various other MAPs. Specifically, applying digestate to lemongrass resulted in an eightfold increase in overall biomass production compared to the control plants (Rowe et al., 2023). In hydroponic systems, utilizing SD as the growing substrate improved nearly all assessed agronomical characteristics in both basil and peppermint compared to agriperlite (Ronga et al., 2018a). Notably, basil achieved the highest dry matter content when solid digestate was combined with liquid digestate (SD + LD). Similarly, for rose-scented geranium (*Pelargonium graveolens* L'Hér.), digestate significantly increased the number of leaves, total branching, plant dry weight, and essential oil content (Calamai et al., 2019).

Chemical fertilizers often lead to the production of high fresh biomass yields (Bufalo et al., 2015). For instance, Burducea et al. (2018) reported a 116% increase in 'Aromat de Buzau' basil under conventional treatment compared to organic fertilizers. However, organic fertilizers lead to the enhancement of key physiological parameters. In particular, field trials using biosolids (an analog to digestate nutrient-rich organic fertilizer) stimulated key physiological traits, including CO_2 assimilation rate and actual PSII quantum yield (Burducea et al., 2018). Therefore, chemical fertilizers may result in the accumulation of biomass; however, organic amendments can lead to increases in quality-related parameters and plant growth.

8.1.2. Physiological mechanisms of digestate action

The beneficial effect of digestates on plant growth is attributed to their diverse nutrient content and the inclusion of biologically active substances (Ronga et al., 2018a). Solid digestate contains macro and micro-nutrients coupled with growth-promoting compounds such as gibberellins, indole acetic acid, and auxin-like molecules (Ronga et al., 2018a; Scaglia et al., 2017). These compounds act as biostimulants, enhancing plant development.

8.2 Impact on essential oil production and composition

8.2.1. Essential oil (EO) yield concentration

Moreover, an inverse relationship is frequently observed between high rates of nitrogen fertilization and EO synthesis, often described as the "dilution effect," where plants prioritize rapid vegetative growth over secondary metabolite production, resulting in a diluted EO content in MAPs (Alami et al., 2024; Kordi et al., 2020).

However, the nature and dosage of the organic amendment, as well as plant type, can dictate the final EO concentration. In particular, applying biosolids at 40 t ha^{-1} was found to increase the EO content of the 'Aromat de Buzau' basil cultivar by 30% (Burducea et al., 2018). In contrast, cultivating sage (*Salvia officinalis* L.) in substrates containing solid digestate as a partial peat replacement resulted in no significant variation in EO yield or overall quality (Greco et al., 2021), indicating that SD can be incorporated without negatively impacting the quality of the marketable products of the crop.

8.2.2. Essential oil chemical profile

Enhanced nutrient availability significantly influences the chemical profile of the secondary metabolites in MAPs. Therefore, digestate and other organic amendments, rich in a diverse range of micronutrients, have the capacity to stir secondary metabolite synthesis towards the production of constituent-specific oils catering to specific industry requirements (Balkrishna et al., 2024; Haddou et al., 2023).

Evidence from hydroponic trials on basil and peppermint indicates that substrates utilizing SD tend to promote sesquiterpene biosynthesis (Ronga et al., 2018a). For basil grown on solid digestate, the key volatile constituent was the sesquiterpene α-bergamotene (reaching up to 69% of the total relative peak area), whereas control and conventional nutrient solutions generally yielded higher concentrations of methyl eugenol (Ronga et al., 2018a).

Biosolids application also affected the EO composition in basil plants. Specifically, it increased the accumulation of desirable components like β-linalool and limonene (Burducea et al., 2018). Furthermore, specific biosolids doses were linked to higher concentrations of eucalyptol, δ-guaiene, τ-cadinol, and γ-cadinene (Burducea et al., 2018). These observed changes underscore the possibility of using digestate to influence secondary metabolite synthesis.

8.3. Digestate effects on substrate and soil properties

8.3.1. Nutrient enhancement and organic matter

Digestate is rich in organic matter (O.M.) and essential plant macro and micro-nutrients, while improving soil health and water retention abilities (Martín-Sanz-Garrido et al., 2025b; Viancelli et al., 2023). The constant addition of digestate increases soil organic carbon (Villarino et al., 2025) and promotes positive modifications to soil structure, such as enhancing the durability and stability of water-stable aggregates (Pranagal et al., 2023).

Moreover, digestate application enhances nutrient availability, notably increasing the extractable concentration of Phosphorus (P) (García-López et al., 2023; Hammerschmiedt et al., 2022) and Potassium (K) (Rolka et al., 2024).

8.3.2. Physical properties and management constraints

While nutrient enrichment is a major benefit, incorporating solid digestate requires addressing specific physicochemical characteristics, which could have a detrimental effect on plant production, such as potential high salinity, high ammonia levels, and shifts in pH (Caspersen et al., 2023; Kovačić et al., 2022b; Zapata-Morales and Moreno-Andrade, 2025).

Studies have shown that properties like dry bulk density and total soil porosity remain stable, under partial peat substitution with SD. However, the substrate's water-holding capacity (WHC) can experience a slight decrease as the proportion of solid digestate increases, though the resulting WHC typically remains within the acceptable range for pot production (Abad et al., 2005; Asp et al., 2022).

From a chemical perspective, digestates are inherently characterized by high pH (often 7.5-8.5) and elevated electrical conductivity (EC) (García-López et al., 2023; Möller and Müller, 2012). High EC is often derived from the salt content in the feedstock, such as swine slurry (Marszałek et al., 2019). Elevated salinity poses an agronomic risk, as EC values approaching or exceeding 2 mS cm^{-1} are regarded as critical thresholds for plant development (Ragályi et al., 2025).

Furthermore, digestate is typically high in ammonium (NH_4^+), which, coupled with a high pH, can generate ammonia NH_3 gas, leading to plant toxicity, reduced growth, and impaired root-to-shoot ratios in plants like basil (Britto and Kronzucker, 2002; Frerichs et al., 2020). Implementing appropriate management techniques is therefore essential. Mitigating salt accumulation can be achieved through appropriate irrigation after digestate application or dilution prior to its use (Ragályi et al., 2025). Moreover, techniques aimed at increasing the conversion of ammonium to nitrate (nitrification) are effective in mitigating ammonium toxicity and simultaneously lowering the pH and ammonia gas emission (Frerichs et al., 2020).

9.1 Heavy metal accumulation in soil from digestate application

Digestate application to agricultural soils raises considerable issues regarding heavy metal (HM) accumulation and environmental safety. Heavy metals can pose significant risks to soil ecosystems, plant health, and human well-being through bioaccumulation in the food chain (Rehman et al., 2021a). Among digestate contaminants, HMs pose particular threats due to their persistence and toxicity to plants, humans, and animals (Czatzkowska et al., 2025). Although AD effectively reduces microbial loads and degrades certain organic contaminants, it does not eliminate HMs, which remain stable throughout the process (Buta et al., 2021).

9.1.1 Heavy metals in digestate: sources and regulatory framework

Heavy metal concentrations in digestate primarily stem from the substrates used for feedstock preparation (Czatzkowska et al., 2025; Peng and Pivato, 2019b). Digestates from the organic fraction of municipal solid waste (OFMSW) contain elevated concentrations of anthropogenic materials compared to agricultural digestates (Baldasso et al., 2023). In intensive livestock systems, copper (Cu), zinc (Zn), and arsenic (As) are used as feed additives, with over 80% of dietary Zn and Cu excreted in active form, making animal manure a significant HM source (Liu et al., 2020; Wan et al., 2024).

Sewage sludge represents another important HM source, commonly containing Cu, Zn, cobalt (Co), iron (Fe), chromium (Cr), lead (Pb), mercury (Hg), nickel (Ni), cadmium (Cd), As, and selenium (Se) (Buta et al., 2021; Czatzkowska et al., 2025). Industrial development significantly increases HM concentrations in wastewater, with surface runoffs and industrial wastewater being primary contamination sources (Fijalkowski et al., 2017). Urban waste typically contains higher HM concentrations, due to industry, construction, and traffic, than rural sources due to increased aerial deposition, with seasonal variations affecting HM content (Kupper et al., 2014).

Studies confirm that Zn appears consistently across all digestates, while Pb is absent from green waste digestates but present in other types (Wolak et al., 2023). Sewage sludge-based digestates show seasonal variation, with spring samples containing higher Cd (1.6 mg kg^{-1} dry matter, DM), Pb (18 mg kg^{-1} DM), Ni (17 mg kg^{-1} DM), Cr (44 mg kg^{-1} DM), and Zn (830 mg kg^{-1} DM) compared to winter samples (Wolak et al., 2023). In contrast, green waste digestates contain minimal HM levels, with Cd at only 0.02-0.03 mg kg^{-1} DM and Zn at 10.4-11.9 mg kg^{-1} DM.

9.1.2 Heavy metal concentrations in various digestates

There is substantial variability in HM concentrations across digestate types (Czatzkowska et al., 2025). Mixed cattle manure and maize silage digestates typically contain 0.9-1.6 mg kg^{-1} Cd, 26-40 mg kg^{-1} Cr, 0.3-0.5 mg kg^{-1} Hg, 12-19 mg kg^{-1} Ni, 8-20 mg kg^{-1} Pb, and 595-790 mg kg^{-1} Zn (Witorożec-Piechnik et al., 2024). Sewage sludge digestates show considerably higher values. Specifically, 2.3-5.4 mg kg^{-1} Cd, 88-91 mg kg^{-1} Cu, and 645-830 mg kg^{-1} Zn (Montusiewicz et al., 2020; Wolak et al., 2023). Sewage sludge combined with food waste digestates shows particularly elevated levels of HM. Specifically, 7.6-10.8 mg kg^{-1} Cd, 144-218 mg kg^{-1} Cr, 1387-2059 mg kg^{-1} Cu, and 3689-5530 mg kg^{-1} Zn (Szaja et al., 2023). Food waste digestates show high variability. In particular, 0.18-5 mg kg^{-1} Cd, 0.02-126 mg kg^{-1} Pb, 0.5-356 mg kg^{-1} Ni, 0.8-4,019 mg kg^{-1} Zn, and 1.4-681 mg kg^{-1} Cu (Ezemagu et al., 2021; Serrano et al., 2020; Løes et al., 2018).

9.1.3 Regulatory framework

The European Union Fertilizing Products Regulation (2019/1009) establishes maximum permissible HM concentrations for organic fertilizers and soil improvers: 1.5 mg kg^{-1} DM for Cd, 2 mg kg^{-1} DM for Cr, 1 mg kg^{-1} DM for Hg, 50 mg kg^{-1} DM for Ni, 120 mg kg^{-1} DM for Pb, 40 mg kg^{-1} DM for As, 300 mg kg^{-1} DM for Cu, and 800 mg kg^{-1} DM for Zn (European Parliament and Council, 2019). Additional EU regulations for soil improvers set limits of 300 mg kg^{-1} for Zn, 200 mg kg^{-1} for Cu, and 100 mg kg^{-1} for Pb (European Commission and Directorate-General for Environment, 2022).

Furthermore, regulatory standards vary substantially between countries. For instance, Denmark enforces strict limits (0.8 mg kg^{-1} for Cd), while Colombia permits up to 80 mg kg^{-1} for the same element (Almeida et al., 2019). This differentiation creates challenges for biogas/digestate producers and highlights the need for consistent international standards. Heavy metals are classified based on biological effects: beneficial at appropriate concentrations (Fe, manganese (Mn), Cu, Zn, molybdenum (Mo)) versus harmful, even at low concentrations (As, Hg, Pb, Cd) (Angon et al., 2024; Rehman et al., 2021b).

9.2 Mobility of heavy metals in digestate-amended soils

9.2.1 Heavy metals mobility

Soil experiments show limited vertical movement of metals. In a 28-day study, researchers observed that Zn, Cu, and Pb concentrations in amended layers (72-108, 28-41, and 24-37 mg kg^{-1}, respectively) were significantly higher than in non-amended layers (16-22, 28-36, and 18-29 mg kg^{-1}) (Baldasso et al., 2025). However, neither sampling time nor pharmaceutical presence significantly affected Zn and Cu distribution in deeper layers, indicating minimal downward movement.

The limited mobility of HM in the soil can stem from several factors. Shaheen et al. (2013) reported that alkaline pH in digestate-amended systems increases soil negative surface charge, favoring cation retention. Additionally, OFMSW digestate presents a lower metal leaching risk than sewage sludge or mineral fertilizers due to the higher mineralized organic matter and lower dissolved organic carbon (DOC) content (Dragicevic et al., 2018; Fang et al., 2017). Long-term studies (90 days) confirmed the downward metal movement to adjacent soil layers, though metals were unable to penetrate deeper in the soil structure (Baldasso et al., 2023).

9.2.2 Metal fractionation and chemical speciation

Heavy metal bioavailability depends on chemical speciation rather than total concentrations. The Bureau Communautaire de Référence (BCR) sequential extraction protocol partitions metals into operationally defined fractions: exchangeable (most mobile), reducible (bound to Fe/Mn oxides), oxidizable (bound to organic matter/sulfides), and residual (least mobile) (Rauret et al., 1999). This fractionation determines mobility, bioavailability, and environmental risk.

Zinc exhibits the highest mobility among commonly studied metals. In amended soils, exchangeable Zn fractions (20-54%) typically exceed those in non-amended soils (4.5-14%), with exchangeable fractions increasing progressively over time while oxidizable-residual fractions decreasing (Baldasso et al., 2025). This contrasts with natural soil aging studies showing decreasing exchangeable forms over time (Sayen et al., 2009; Sayen and Guillon, 2014). Increased Zn bioavailability leads to the adsorption of Zn to Fe/Mn oxides under alkaline conditions and complexation with labile organic molecules that degrade over time (Bradl, 2004; Gu and Bai, 2018; Zheljazkov and Warman, 2004).

Copper demonstrates the lowest mobility, with 82-94% in oxidizable-residual fractions in amended soils. This reflects Cu's tendency to form stable organic matter complexes under alkaline conditions and strong adsorption to silicate minerals, while appearing to be markedly more mobile under acidic conditions (Gu and Bai, 2018; Lockwood et al., 2015). Temporal increases in reducible Cu fractions result from organic matter mineralization, releasing cations that react with metal oxides (Pan et al., 2021).

Lead (Pb) in amended soils shows 31-92% fractionation in reducible fractions compared to 3-20% in deeper layers. Over time, exchangeable and reducible Pb fractions increase while oxidizable-residual fractions decrease. Lead behavior is governed by adsorption onto Mn, Fe, and aluminum (Al) oxides through a two-phase process: rapid initial adsorption followed by diffusion into mineral particles (Bradl, 2004). In deeper layers, metals remain primarily in residual fractions, indicating limited redistribution and maintenance of immobile forms due to strong bonds with clay minerals (kaolinite) and silicates (muscovite) (Baldasso et al., 2023).

9.2.3 Comparison of fresh and granulated digestate

Digestate can be applied as fresh liquid, solid fraction, or processed granules. Granulated digestate, produced through dehydration, grinding, and roller pressing into pellets, has approximately 95% DM versus 5% for fresh digestate (Bartkowiak and Piątek, 2020).

Pot experiments comparing fresh and granulated digestate from maize silage with distillers' solubles or pig slurry have demonstrated that the maximum permitted HM limits were not exceeded in any treatment, confirming granulated digestate’s safety for agricultural use (Bartkowiak and Piątek, 2020). Moreover, the researchers demonstrated that fresh digestate application results in statistically lower Zn, Cu, and Mn contents in the soil compared to soils amended with distillers' solubles or pig slurry. Soils treated with granulated digestate exhibited even lower Cu, Fe, and Cd concentrations, while soils treated with lime showed substantially reduced Cd concentrations (0.11-0.15 mg kg^{-1}), compared to NPK-only fertilized plants (Bartkowiak and Piątek, 2020).

9.3 Influence of soil management on heavy metal dynamics

9.3.1 pH effects

Trace metals exhibit higher solubility at low pH and redox potential, increasing mobility and bioavailability (Cipullo et al., 2018). Alkaline conditions in digestate-amended systems (pH ~8.6) increase negative surface charge, favoring cation retention (Shaheen et al., 2013). Notably, liming considerably reduces Cd concentrations and improves Zn immobilization, demonstrating the importance of pH management in managing HM mobility (Bartkowiak and Piątek, 2020).

9.3.2 Mineral fertilization versus digestate

In several cases, NPK fertilization resulted in higher metal concentrations than non-fertilized treatments, owing to trace metals present as impurities in NPK fertilizers (Czarnecki and

Düring, 2015; Milinović et al., 2008; Wyszkowski and Brodowska, 2021). However, there is no clear connection between NPK fertilization and increased HM concentration. Bartkowiak and Piątek (2020) performed a comparative analysis, revealing that digestate application can result in lower heavy metal concentration than mineral fertilization, suggesting that digestate-based fertilization may offer advantages for limiting HM accumulation.

9.4 Environmental implications and risk assessment

9.4.1 Accumulation potential and ecological risk

Experimental evidence indicates that digestate application leads to limited short-term HM accumulation potential, with total metal concentrations remaining below regulatory thresholds and minimal infiltration to deeper layers (Baldasso et al., 2023, 2025; Bartkowiak and Piątek, 2020). However, long-term digestate addition could cause heavy metal pollution. Therefore, monitoring of HM concentration in the soil is imperative to ensure soil health.

According to Czatzkowska et al. (2025), ecological risk assessments using geo-accumulation index (Igeo) and ecological risk factor (Er) revealed that 25-33% of analyzed digestates are extremely contaminated with Cd and Hg (Igeo > 5), and 45-50% pose very high ecological risk (Er > 320) for these metals. High soil HM concentrations lead to biodiversity loss and ecosystem dysfunction (Hattab-Hambli et al., 2020; Jia et al., 2022). Continuous organic amendment fertilization affects HM bioavailability and fractionation, with residual levels absorbed by roots and accumulated in edible plant parts even at low concentrations (Buta et al., 2021; Jia et al., 2022).

9.4.2 Comparison with alternative amendments

Compared to other biosolids, OFMSW digestate reduces metal leaching risk due to higher mineralized organic matter and lower DOC (Fang et al., 2017; Shaheen et al., 2013). Digestate from various sources exhibits lower metal leaching compared to sewage sludge and mineral fertilizers, even during intense precipitation (Dragicevic et al., 2018, 2017). The heavy metal content in digestates can be comparable to or lower than composts, depending on the substrates used for their production (Kupper et al., 2014). However, metals and other pollutants remain in the digester after the AD process has been finalized, while the adsorption of HM to organic matter can further increase the HM content of the digestate, especially in the solid fraction (Golovko et al., 2022; Wei et al., 2024). Digestate chemical composition resembles other sustainable fertilizers (manure, slurry) with a low carbon-to-nitrogen ratio, which facilitates rapid nitrogen mineralization and plant availability (Kowalczyk-Juśko and Szymańska, 2015; Alburquerque et al., 2012).

9.4.3 Heavy metal risk assessment

Among digestate metals, Zn presents the highest mobility and environmental concern, though concentrations typically remain within acceptable ranges (Akhtar et al., 2025). Cu shows low mobility due to stable organic matter complexes and silicate affinity (Gu and Bai, 2018). Pb demonstrates increasing mobility over time through adsorption to Fe, Mn, and Al oxides, though total concentrations typically remain low (Bradl, 2004). Cd requires particular attention, given its high toxicity, mobility, and bioaccumulation tendency. Moreover, Cd adversely affects soil biological activity, plant metabolism, and animal/human health, posing a significant environmental concern (Khan et al., 2017; Rasin et al., 2025).

10.1 Pathogen translocation to soil and plant tissue from digestate use

The management of digestate remains of critical concern, particularly regarding its biosafety when applied to agricultural lands. The substrates commonly used in anaerobic digestion, including food waste, sewage sludge, and livestock manure, are usually filled with numerous and diverse pathogenic microorganisms (Jiang et al., 2020a). Analysis of sludge samples from Spanish wastewater treatment plants revealed that *Cryptosporidium* oocysts and *Giardia* cysts were detected in 87% of raw sludge samples and 100% of treated samples (Amorós et al., 2016). Similarly, surveys conducted on Swedish wastewater treatment plants indicated that 67% of raw sludge samples and 55% of treated samples were positive for *Salmonella* (Sahlström et al., 2004).

Farmyard manure and slurry, which constitute primary feedstocks for anaerobic digestion plants, may contain elevated concentrations of pathogens, and complete inactivation through the AD process remains unlikely (Hutchison et al., 2004). Foodborne illness and gastroenteritis represent global health concerns, with the application of unprocessed animal slurry and anaerobic digestate on agricultural land potentially contributing to pathogen transmission to humans through the food chain (Clarke et al., 2017; Torgerson et al., 2015).

10.1.1 Gram-negative bacteria

Gram-negative bacteria represent one of the most significant groups of pathogens found in digestate. The most prevalent species include *Escherichia coli*, *Salmonella* (Jiang et al., 2018), *Listeria* (Trząskowska et al., 2018), *Yersinia* (Massé et al., 2011), and *Campylobacter* (Manyi-Loh et al., 2016). These bacteria possess a cell wall structure consisting of a sole lipid-rich outer membrane and a monolayer of peptidoglycan, which offers relatively limited protection against environmental stress factors (Beveridge, 1999). Consequently, Gram-negative bacteria are generally sensitive to thermophilic temperatures, volatile fatty acids, and ammonia, exhibiting relatively low resistance compared to other pathogen types (Himathongkham et al., 2000; Watcharasukarn et al., 2009).

Salmonella represents a particularly concerning pathogen due to its ability to survive for extended periods in manure during storage. Research has demonstrated that *Salmonella* can survive for as long as 84 days in pig manure in storage conditions (McCarthy et al., 2015). Moreover, *Salmonella* has been detected in manure from half of the 30 Irish pig farms sampled by McCarthy et al. (2013), with most isolates being multi-drug resistant *S. Typhimurium*. Non-typhoidal *Salmonella* causes salmonellosis, resulting in acute gastroenteritis, diarrhea, fever, abdominal cramps, reactive arthritis, and post-infectious irritable bowel syndrome (Manyi-Loh et al., 2016; Scallan et al., 2015).

E. coli, particularly the O157:H7 serotype, causes acute gastroenteritis, diarrhea, vomiting, hemolytic uremic syndrome, abdominal cramps, and hemorrhagic colitis (Manyi-Loh et al., 2016; Scallan et al., 2015). The Foodborne Diseases Active Surveillance Network reported 24,484 illnesses, 5,677 hospitalizations, and 122 deaths in 10 US sites in 2017 (Marder Mph et al., 2018), while 360,524 human cases, 34,442 hospitalizations, and 156 deaths were reported in 37 European countries in 2016 (EFSA and ECDC, 2017). Human norovirus derives most commonly from food waste and is unlikely to be found in farmyard manure and slurry (Mattison et al., 2007). However, the co-digestion of food waste with animal slurry may introduce this pathogen in the digestate.

10.1.2 Gram-positive bacteria

Gram-positive bacteria found in digestate include both non-spore-forming species, such as *Enterococcus*, and spore-forming species, such as *Clostridium* (Manyi-Loh et al., 2016). These bacteria possess a cell wall that is considerably thicker than that of Gram-negative bacteria, consisting of teichoic acids and highly cross-linked, multi-layered peptidoglycan. This structure serves not only as a component of the cell membrane but also functions as a permeability barrier, preventing toxic chemicals from penetrating the cell (Dijkstra and Keck, 1996). Consequently, Gram-positive bacteria exhibit greater resistance to various stress conditions and are able to survive at a wide range of pH values (4.6-9.9), temperatures (5-50 °C), free volatile fatty acid concentrations (up to 375 mg L^{-1}), and osmotic pressures (up to 40% bile salts and 6.5% NaCl) (Fisher and Phillips, 2009; Jiang et al., 2018).

Clostridium perfringens presents a particular challenge as it can form highly resistant spores. While the vegetative cells of *Clostridium perfringens* are sensitive to thermophilic temperatures, the spores exhibit remarkable heat resistance. Research by Byrne et al. (2006) reported that the decimal reduction time of vegetative cells was 16.3 min and 0.9 min at 55 °C and 65 °C, respectively, whereas the decimal reduction time of *Bacillus cereus* spores was 34.2 min and 2.2 min at 90 °C and 100 °C, respectively. Under mild environmental conditions, these spores can survive for many years (Sahlström, 2003). *Clostridium perfringens* causes acute gastroenteritis, tetanus, botulism, and black leg disease (Manyi-Loh et al., 2016). Spore-forming organisms, which commonly occur in animal wastes (Snell-Castro et al., 2005), are not usually adequately reduced during anaerobic digestion (Bagge et al., 2005; Sahlström et al., 2004). *Clostridium* spp. spores demonstrate remarkable heat resistance, requiring temperatures of 70 °C to 110 °C to inactivate vegetative cells and spores, respectively (Talukdar et al., 2016).

10.3 Regulatory standards for pathogens in digestate

To assess the biosafety of digestate for agricultural application, analysis of indicator organisms is essential. Biosafety standards vary considerably between countries and regulatory jurisdictions. The most commonly specified indicator bacteria for Gram-negative bacteria are *Salmonella*, *E. coli*, and faecal coliforms.

In the United Kingdom, *E. coli* is required to be less than 10^3 cfu g^{-1} in fresh matter, and *Salmonella* spp. must be absent in 50 g of fresh matter (BSI PAS110, 2014). The European Union and Ireland require *E. coli* to be less than 10^3 cfu g^{-1} in fresh matter, *Enterococcaceae* to be less than 10^3 cfu g^{-1} in fresh matter, and *Salmonella* to be absent in 25 g of fresh matter. Additionally, EU regulations specify minimum $\log_{10}$ reductions of ≥ 5 for *Enterococcus faecalis* and *Salmonella Senftenberg*, ≥ 3 for Parvovirus, and ≥ 3 for *Ascaris* eggs (EU Commission, 2011; IrBEA, 2012). Irish regulations specify that thermal and chemical processes must reduce indicator bacteria *Enterococcus faecalis* or thermo-resistant *Salmonella Senftenberg* by at least five $\log_{10}$ and thermo-resistant parvovirus by three $\log_{10}$.

In the United States, class A biosolids standards require faecal coliforms to be less than 10^3 mpn g^{-1} in total solids (TS), *Salmonella* less than 3 mpn $4g^{-1}$ TS, *Helminth* less than 1 viable egg 4 g^{-1} TS, and enteric viruses less than 1 pfu $4g^{-1}$ TS. Class B standards permit faecal coliforms up to 2×10^6 mpn g^{-1} TS (US EPA, 1994). Chinese regulations require faecal coliforms to be less than 10^4 mpn g^{-1} fresh matter in ambient and mesophilic AD systems and less than 10^2 mpn g^{-1} in fresh matter in thermophilic AD systems, *Salmonella* absent in 10 g of fresh matter, and $\geq 95\%$ reduction in *Ascaris* eggs (Cheng et al., 2024).

For Gram-positive bacteria, the family Enterococcaceae is required to be less than 10^3 cfu g^{-1} in fresh matter in the EU. Several European countries have established comprehensive standards governing digestate quality and use, including the United Kingdom (BSI PAS110, 2014), Germany (RAL GZ 245), Sweden (SPRC120), Belgium (VLAREMA), Austria (ARGE), Switzerland (VKS-ASIC), Denmark (EC No. 834), and France (Standard NF U44-051), which have implemented specifications for physicochemical characteristics, system management, and contaminant concentration limits (Al Seadi et al., 2013).

10.4 Pathogen survival in soil following digestate application

10.4.1 Environmental factors affecting pathogen survival

Following application of digestate to agricultural land, pathogen survival in soil is influenced by multiple environmental factors. Temperature represents one of the most critical factors affecting pathogen persistence. The inactivation rates of various pathogens at ambient and mesophilic conditions are very low, with thermophilic temperatures exhibiting a higher efficiency of pathogen elimination (Jiang et al., 2020a). For instance, the inactivation rate constants for *E. coli* were reported as 0.12 d^{-1} at ambient temperatures (20-25 °C), 0.13 d^{-1} at mesophilic temperatures (30-40 °C), and 2.01 d^{-1} at thermophilic temperatures (50-55 °C) (Pandey and Soupir, 2011). Similarly, for *Enterococcus*, the inactivation rate constants were approximately 0 at ambient and mesophilic temperatures but ranged from 1.0 to 6.7 d^{-1} at thermophilic temperatures (Ziemba and Peccia, 2011).

The effect of temperature on pathogen survival is both direct and indirect. Particularly, temperature directly affects the cell membrane composition of pathogens. Increasing temperature from 37 °C to 70 °C increases membrane fluidity and permeability by changing the membrane lipid phase, denaturing membrane proteins, and collapsing cytoskeletal proteins (Fan and Evans, 2015). This enables more rapid diffusion of toxic compounds into the cytoplasm and cell growth inhibition (Salsali et al., 2006).

Soil pH significantly influences pathogen survival by affecting the ionization state of organic acids and ammonia, which in turn determines their antimicrobial activity. Free volatile fatty acids are considered more toxic to pathogens than ionized volatile fatty acids because they are lipophilic and can permeate the cell membrane freely (Lin et al., 2022). At neutral pH, volatile fatty acids cause little harm to pathogens, while their toxicity increases considerably when pH decreases below 7 due to the accumulation of free volatile fatty acids (Otite et al., 2024). Similarly, free ammonia (NH_3) is considered more toxic to pathogens than ammonium ion (NH_4^+) due to its lipophilic properties, enabling NH_3 to pass through cell membranes freely (Dravecka et al., 2025).

10.4.2 Pathogen survival characteristics

The resistance of different pathogen types to environmental stress factors varies considerably based on their physiological structures and characteristics. According to Jiang et al. (2020a), statistical analysis of 141 published studies indicated that the resistance of pathogens follows the order: spore-forming Gram-positive bacteria > non-spore-forming Gram-positive bacteria > nematode ≥ Gram-negative bacteria ≥ viruses.

Gram-negative bacteria such as *E. coli* and *Salmonella* can be reduced to below the limit of detection within 4 days and 14 days in batch and continuous thermophilic digesters, respectively. However, under mesophilic conditions, these pathogens may persist for extended periods (Wang et al., 2025). In wet mesophilic AD systems, *E. coli* can be reduced to below 10^3 cfu g^{-1} after 40-60 days, while a shorter HRT of 20 days resulted in limited reductions (Smith et al., 2005). *Salmonella* survival is particularly concerning, with the pathogen persisting for 60 days or more under mesophilic conditions without effective inactivation (Fongaro et al., 2018).

E. coli typically does not survive longer than several days to weeks in soil, although some researchers have detected survival periods of up to 30-68 days under saturated conditions or after the application of pig manure (Unc and Goss, 2004). Certain *Salmonella* species have been found to persist up to 110 days (Mawdsley et al., 1995), while *Listeria* may survive up to 128 days (Venglovsky et al., 2009). Furthermore, some researchers have reported that the application of cattle manure to soil can result in *E. coli* O157 and *Salmonella* remaining in the soil for up to one month under field conditions (Nicholson et al., 2005).

Ascaris eggs demonstrate remarkable environmental persistence due to their protective eggshell structure. Research indicates that *Ascaris* eggs cannot be effectively inactivated at temperatures below 40 °C in the absence of other stress factors (Pecson and Nelson, 2005). At 20-30 °C for 24-80 days, only minor reductions were observed (Pecson et al., 2007). At 37 °C, only a 75% reduction was achieved after 10 days (Kato et al., 2003). However, at temperatures of 40 °C and above, *Ascaris* eggs can be decreased to below the limit of detection within 12 days (Pecson et al., 2007), and ≥ 95% reduction can be obtained at 47 °C within 2 days (Kato et al., 2003).

10.4.3 Role of indigenous soil microbiota in pathogen suppression

The indigenous soil microbiota plays a critical role in suppressing allochthonous pathogens introduced through organic amendments. Potential pathogens supplied with amendments proliferate more readily in sterilized soils lacking autochthonous microbiota, demonstrating that pathogen growth is enhanced under conditions of limited competition (Priyadarshini et al., 2025; Wang et al., 2024). Research has shown that digestate-borne potential pathogenic bacteria, such as *E. coli* and *Enterococcus faecium*, did not increase when digestate was applied in non-sterile soil, suggesting that soil microorganisms can inhibit the establishment and growth of digestate-borne pathogens (Gong et al., 2023).

Microbial competition between indigenous soil microbes and pathogens has been demonstrated in laboratory experiments using co-cultures. Buchanan and Bagi (1999) showed that *Pseudomonas fluorescens* limits the growth of *L. monocytogenes*, with the intensity of interaction depending on environmental conditions, including temperature and sodium chloride concentration. *Salmonella typhimurium* and *S. newport* grew well in irradiated material but died in non-sterilized compost, suggesting suppression by autochthonous compost microbiota (Hussong et al., 1985; Sidhu et al., 2001).

Soil microbes suppress pathogens through several mechanisms, including antibiotic production, nutrient deprivation mediated by chelators, and interference with pathogenicity factors (Haas and Défago, 2005). Petras and Casida (1985) inoculated spores of *Bacillus thuringiensis* in soil and found that spores survived but lacked the ability to germinate, suggesting sporostatic effects mediated by microorganisms, secondary metabolites, or extracellular enzymes. In contrast, *Salmonella* was present at very low levels in soils amended with anaerobically digested biosolids, becoming undetectable two weeks after amendment application (Zaleski et al., 2005a).

10.5 Mechanisms of pathogen translocation to plant tissue

10.5.1 Pathways of pathogen transfer

When digestate is applied to agricultural lands, pathogens may transfer to soil, water, plants, animals, and humans, leading to serious public health concerns (Senevirathne and Kaparaju, 2025b). The translocation of pathogens from digestate and other organic fertilizers to plant tissue can occur through multiple pathways. Direct contact between contaminated soil and above-ground plant tissue, particularly leafy vegetables and root crops, represents a primary route of contamination (Fletcher et al., 2013; Solomon et al., 2002). Additionally, pathogens may be internalized by plants through root uptake and subsequent translocation to edible plant parts (Fletcher et al., 2013).

The recurrence and persistence of pathogens after digestate treatment present additional challenges. Research has demonstrated that thermophilic-inactivated faecal coliforms can reappear after the thermophilic process has finalized (Riau et al., 2010). This resuscitation of faecal coliforms has also been observed in post-treatment processes, including centrifugation, transport, storage, and land application (Gardner and Örmeci, 2010; Iranpour and Cox, 2006). The recurrence may be attributed to incomplete inactivation during thermophilic digestion, contamination by mesophilic biosolids or post-treatment facilities, and revival of faecal coliforms due to large temperature drops. Pathogen regrowth during digestate storage represents a serious issue (Carraturo et al., 2022; Jiang et al., 2020b), potentially resulting from non-hygienic conditions in storage and transport tanks (Carraturo et al., 2022) or reinoculation by animal vectors (Zaleski et al., 2005b).

The low solid content of digestate compared to fresh manure may result in higher mobility of any microbes supplied, facilitating their movement through soil and potentially to plant root zones (Unc and Goss, 2004). Factors affecting pathogen transfer to plants include irrigation method, rainfall intensity, soil texture, plant architecture, and time between final amendment application and harvest. Girardin et al. (2005) investigated the behaviour of pathogen surrogates *Listeria innocua* and *Clostridium sporogenes* during the production of parsley in fields fertilized with contaminated amendments, demonstrating that even minimal soil contamination can result in plant tissue contamination through splash effects during irrigation or rainfall.

10.5.2 Crop contamination risks

Different crop types present varying levels of risk for pathogen contamination. Root vegetables and leafy greens that have direct soil contact are particularly vulnerable to pathogen transfer. The initial pathogen concentration in the applied digestate significantly affects the contamination risk of harvested produce. Studies have demonstrated that even when digestate meets regulatory standards, detectable levels of pathogens may remain that can potentially transfer to crops under certain conditions (Nag et al., 2021).

Ready-to-eat crops pose a particular concern when consumed without further treatment or processing (Mawdsley et al., 1995). The number of *Listeria* bacteria remaining in amended soils can be extremely high, which is concerning given that as few as one colony-forming unit of *Listeria* per gram of soil can be transferred to vegetable leaves by splashing during rainfall or irrigation (Girardin et al., 2005). Molecular detection methods can distinguish between pathogenic *L. monocytogenes* and the harmless soil *L. innocua*, which shares morphological, biochemical, and molecular features with its pathogenic relative (Liu et al., 2003).

Aromatic plants and herbs, which are often consumed raw or with minimal processing, demand particular attention when cultivated with digestate fertilization (Carraturo et al., 2022; Sobhi et al., 2024). The essential oil content of these plants may provide some antimicrobial protection, but pathogen contamination risks must still be carefully managed through appropriate digestate treatment and application practices (Boy et al., 2023). The timing of digestate application relative to harvest, the method of application (soil incorporation versus surface spreading), and post-harvest handling practices all influence the final pathogen load on harvested crops (Korba et al., 2024; Piveteau et al., 2022).

10.6 Risk mitigation strategies

10.6.1 Optimization of anaerobic digestion process parameters

Optimization of anaerobic digestion operating conditions is essential for achieving energy recovery, pathogen inactivation, and biowaste stabilization. Pathogen inactivation efficiency is significantly influenced by pathogen type, temperature, and operational conditions (Jiang et al., 2020b). Thermophilic temperatures (50-55 °C) can effectively inactivate most pathogens, whereas mesophilic temperatures are not effective for pathogen inactivation, regardless of hydraulic retention time (Jiang et al., 2020c).

High ammonia contents in manure-digesting reactors may further promote product sanitation (Ottoson et al., 2008). Temperature and HRT represent the primary inactivation factors during AD (Thomas et al., 2019), while residence time and volatile fatty acid concentrations influence pathogen survival post-application.

Dry anaerobic digestion represents a promising strategy for pathogen inactivation due to the accumulation of volatile fatty acids and ammonia. Unlike wet digestion, dry mesophilic AD can eliminate both Gram-negative and Gram-positive bacteria effectively within 4-7 days due to the accumulation of intermediate products (Jiang et al., 2018; Orzi et al., 2015). For example, dry mesophilic AD can reduce *E. coli* to below the limit of detection within 4-7 days due to the inhibition by high volatile fatty acid concentrations (Wang et al., 2025b; Jiang et al., 2018).

Pre-AD pasteurization proves more effective for *Cryptosporidium parvum*, *norovirus*, and *Mycobacterium thermoresistibile*, while post-AD pasteurization most reliably ensures digestate safety when following appropriate protocols. The Irish government's legislation states that digestate has to be pasteurized at 60 °C for four days prior to application, while the EU directives require that digestate must be pasteurized at 70 °C for one hour, highlighting that national legislation can be stricter compared to EU directives (Coultry et al., 2013). The C:N ratio of the feedstock also affects pathogen inactivation during digestion, with higher C:N ratios generally resulting in greater log reductions for coliform bacteria, *E. coli*, and *Mycobacterium thermoresistibile* (Gurtler et al., 2018).

Pasteurization process is highly recommended for mesophilic AD plants, prior to land application or other use, to ensure adequate pathogen reduction. Research by Pulvirenti et al. (2015) demonstrated that pelleting constitutes a successful method of eliminating *Clostridium* species from biogas plant digestates, addressing one of the most important microbiological safety concerns associated with digestate application.

10.6.2 Digestate application management practices

Appropriate management of digestate application can significantly reduce pathogen translocation risks. Incorporation of digestate into soil rather than surface application reduces direct contact between pathogens and above-ground plant parts while promoting pathogen reduction through increased exposure to soil microbial competition and adverse environmental conditions (Chojnacka and Moustakas, 2024).

Evidence suggests that a delay of approximately 90 days between land application of organic amendments and crop harvesting represents a relatively safe interval. After three months, the number of potential pathogens in amended soils typically does not exceed that in control soils (Venglovsky et al., 2009). However, pathogen survival in soils depends on numerous factors, including environmental conditions, soil pH, soil nutrient status, the quality of waste applied, and the microbial species involved (Moynihan et al., 2015).

Selection of appropriate crops for digestate fertilization presents another important risk mitigation strategy. Crops that undergo processing or cooking before consumption present a lower risk than those consumed raw. When digestate is applied to fields producing crops that are consumed raw, plant disinfection protocols and longer waiting periods between application and harvest should be implemented.

Storage of digestate represents another effective method for reducing microbial presence, particularly for non-spore-forming bacteria such as faecal coliforms and *Salmonella* spp. (Nag et al., 2021). However, spore-forming *Clostridium perfringens* showed no inactivation during two-year storage studies at 35-37 °C, indicating that additional treatments, such as low-dose alkaline disinfection with calcium oxide, may be required for complete elimination of these organisms (Bujoczek et al., 2002). Pascual-Benito et al. (2015) assessed the persistence of *E. coli*, clostridial spores, and *Salmonella* spp. in mesophilic and thermophilic produced digestates after different pasteurisation treatments and storage conditions were implemented. Results showed that pasteurization at 55 °C did not entirely eliminate *E. coli* pathogens, with injured *E. coli* bacteria resuscitating after 24 hours. Different sludge treatments altered the inactivation kinetics of *E. coli*, while *Clostridium* spp. showed lower mortality rates. Moreover, regrowth of *Clostridium* spp. was observed in stored sludge for up to 60 days. Conversely, no regrowth was observed for *Salmonella* spp.

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Chapter 2: Reports on the results from the biogas units and the scientific community questionnaires

2.1 Report on the substrates used by the biogas production plants

The present survey was stratified and included biogas power plants in Central Macedonia and Epirus (Figure 1). Of the 13 biogas power plants in the two regions in 2022, 10 participated in the current survey.

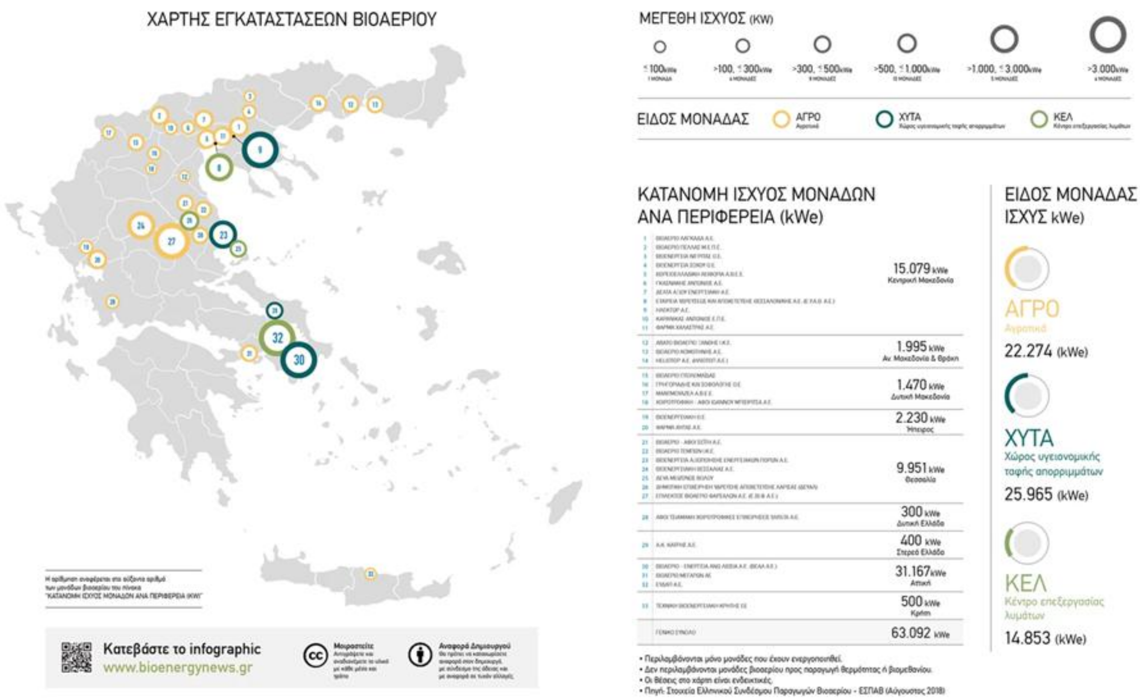

Figure 1. Map of biogas power plants in Greece in 2018 (Source: https://bioenergynews.gr/infographics).

The survey mainly focused on the different types of agricultural substrates used in biogas power plants for energy production. The participants' awareness regarding recent European Directives and the cost of transportation and raw material acquisition was also examined.

Most plants had an energy production capacity of 1MW, with two plants having considerably lower capacity (12 KW and 500 KW) and two more considerably higher (1.56MW and 3MW) (Table 1). Most survey participants held a managerial position (Production Manager, Plant Manager, Owner, etc.). 70% of respondents used slurry from cattle, while only 30% from swine. The majority of the participants stated that they use poultry waste (80%), though in small quantities (0-5%). Synchronously, they also reported using other substrates not included in the survey, such as cheese whey, olive mill and cotton processing residues, at a percentage ranging from 5 to 25%.

Table 1. Name, location, and power generation capacity of the biogas power plants participating in our survey.

Biogas power plant	Location	Power generation capacity (MW)
Farma chalastras	Chalastra, Thessaloniki	1
Biogas Lagada	Lagadas, Thessaloniki	1
Biogas Nigrita	Nigrita, Serres	1
Bioenergy Sochou	Sochos, Thessaloniki	0,95

Heliotop	Agios Athanasios, Drama	0,5
Epirus energy	Epirus	1,56
Grigoriadis and Sofologis	Kozani	0,12
Farma Chytas	Filippiada, Preveza	3
Vorioelladiki aeiforia	Thessaloniki	1
Biogas Pella	Pella	1

Regarding the use of plant matter, beyond cereal crops (such as maize and triticale), most respondents answered that they use fruits (75%) and vegetables (50%). Tubers, bulbs, or roots are used by approximately 40% of participants. Furthermore, participants stated that they also use plant substrates other than those listed in the survey, such as cotton processing residues, food industry waste, spent animal feed, and rice flour, though in limited quantities. In general, substrate type depended mainly on seasonal availability and cost of acquisition.

Moreover, 40% of respondents stated that they use cereal crops in their reactors. 50% stated that they use corn silage, 25% barley, and the remaining 25% sorghum. Furthermore, cereal crops represent 5-25% of the anaerobic digestion feedstock, and their use depends on the season and availability in the area.

Additionally, 90% stated that they do not use residues from cereal crops, except for 10% who reported using rice cultivation residues. Nevertheless, all participants responded positively when asked if they considered rice cultivation residues as a potential feedstock for biogas production. They were also positive about being notified regarding novel processing methodologies, which would greatly enhance rice cultivation residues' methanogenic potential. Additionally, all the responders were positive about using rice straw as feedstock in their reactors.

Furthermore, all participants stated that they are aware of the EU's goal to transition into a climate-neutral economy by 2050, while 90% knew that, according to the COM (2020) directive, there will be a need for a 29% reduction in methane emissions by 2030. In contrast, only 30% stated that they knew the Renewable Energy Directive II (RED II), which provides disincentives for cultivating plants with the sole purpose of converting them into energy and incentives for using agricultural residues.

Finally, 50% of the respondents stated that their suppliers are at a distance greater than 10 km, while 40% claimed that the average distance varies between 5 and 10 km. The cost of feedstock transportation was proportional to the distance, ranging from 0.4 euros km^{-1} to 5 euros km^{-1}. Consequently, all participants stated that their suppliers needed to be in close proximity. The cost of purchasing silage from cereal crops, according to the responders, is significantly high, ranging from 30 to 80 euros ton^{-1}.

2.2 Report on the results from the scientific community and local government

In the present survey, 11 participants took part, most of whom were researchers and scientists, while all of them belonged to policy-making bodies. Specifically, the majority stated that they were either researchers (45.5%) or scientists (36.4%), while approximately 20% (18.2%) stated they are members of the regional government. 75% stated that they know the RepowerEU plan and believe that it will positively affect Greece's energy mix, mainly because it will promote the construction of renewable energy plants within agricultural facilities. Moreover, the majority (75%) commented positively on the EU's goal to double biogas production by 2030, with a small percentage expressing reservations (8.3%) concerning the balance between energy self-sufficiency and food security. Over 90% (90.9%) of participants believed that the war in Ukraine would lead to an increase in biogas production plants within the EU, and especially in Greece. All respondents stated that biogas production constitutes a sustainable methodology for treating wastes that promotes European and domestic energy self-sufficiency and independence.

When asked if they knew the RED II directive, most (81.8%) replied negatively, but once informed, they commented positively on the directive's approach to provide incentives to promote the use of agricultural residues instead of cultivating plants solely for energy production. Also, 81.8% stated that through this directive, more agricultural land will be available for the production of agricultural products, and thus enhance food security in the EU. Over 60% (63.6%) of the respondents stated that they are aware of the COM 663 directive of 2020, and more than 80% (81.8%) knew that this directive mandates that the EU has to reduce methane emissions by 29% by 2030. Moreover, 54.5% stated that, via this directive, targeted support will be given to ensure that biogas is produced from sustainable sources such as agricultural residues. In addition, over 90% (90.9%) supported the notion that rice cultivation residues should be utilized to facilitate the EU's overarching goal of reducing CH_4 emissions by 29% by 2030.

All participants agreed that digestate has a major role to play in agricultural production in the future. Moreover, the majority (83.4%) stated that anaerobic digestion appears to be the most efficient way of utilizing rice cultivation residues. 91.7% agreed with the EU's intention to include digestate in the exhaustive list of organic fertilizers with CE certification, while all of the participants considered that this move would greatly increase the use of digestate as organic fertilizer. All respondents agreed that the use of digestate enhances the circular economy within the European Union and that it could be an additional income for biogas production plants. Ultimately, all participants responded that an agreement between farmers and biogas power plants to exchange agricultural residues for digestate would be feasible and beneficial for all the stakeholders involved.

Chapter 3: Enhancing methane production from rice crop residues via pretreatments and co-digestion with cattle or swine slurry

Sections of this chapter have been published in:

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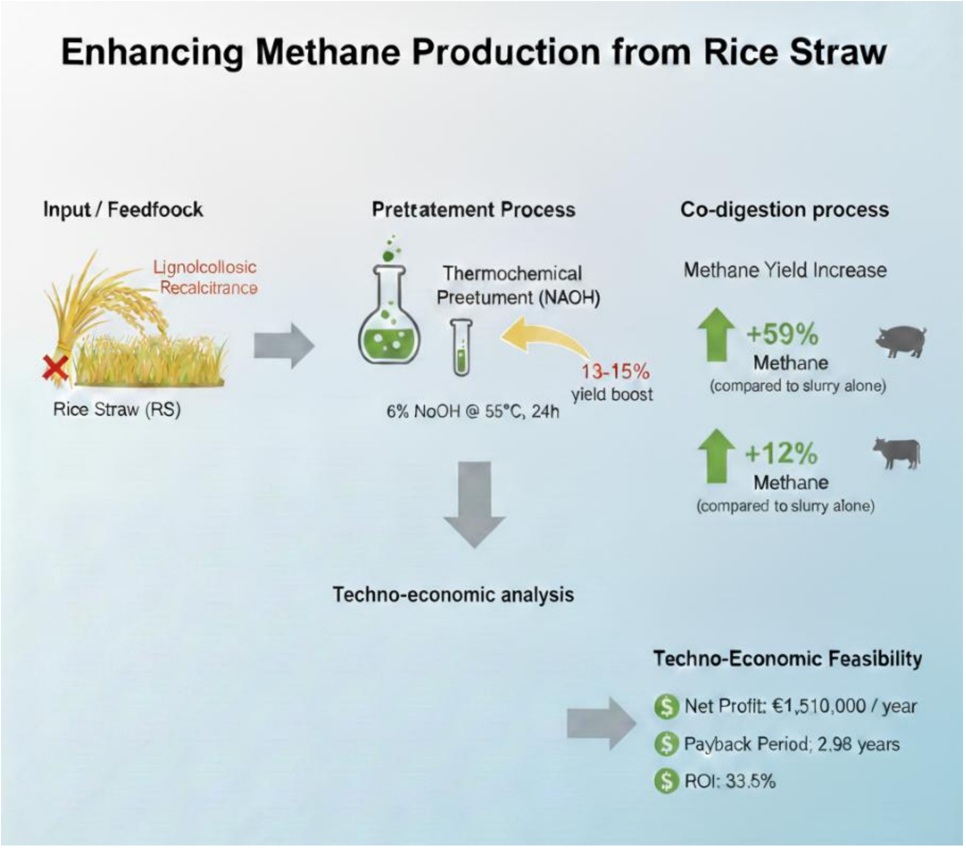

Abstract

Improper management of residues from rice cultivation and livestock farming can exert severe pressure on the environment. Anaerobically digesting these residues for biogas production is a plausible solution to mitigate their environmental impact. However, due to the low degradability of rice straw, pretreatment is imperative to enhance its methane production. Therefore, this study investigated the effects of eight thermal and four thermochemical pretreatment methods on the biodegradability of rice straw. Results indicated that the thermochemical pretreatment, with NaOH, increased methane production by 13%-15%, while thermal pretreatment increased it by 2%-5%, compared to untreated rice straw. Subsequently, the most effective pretreatment was used in co-digestion batch experiments with cattle and swine slurries separately to determine the optimal ratio of pretreated rice straw (p-RS) with each slurry. Co-digestion of p-RS with swine slurry increased methane production up to 59%, while co-digestion with cattle slurry increased it by 14%, compared to the mono-digestion of each slurry. Moreover, a techno-economic analysis was employed to assess the feasibility of establishing a biogas power plant treating the optimum co-digestion mixture. Based on our assessment, the plant can generate an annual revenue and net profit of approximately €2,418,000 and €1,510,000, respectively. The payback period was estimated at 2.98 years, while the return on investment and profit margin were 33.5% and 62.4% per year, respectively.

1. Introduction

The world population will increase to 10.3 billion by the end of the century (United Nations, 2024), necessitating greater food production. Simultaneously, the competition for arable land between energy crops cultivated for energy production and food crops can lead to instability in food prices (Boly and Sanou, 2022). In contrast, agricultural residues represent an underexploited resource that does not compete with food production (Korres et al., 2023). Specifically, approximately 517.4 million tons year^{-1} of maize and wheat straw globally can be utilized to generate 7.2 EJ of energy through anaerobic digestion (Korres et al., 2023). The potential to recover energy from agricultural residues has led the EU to provide incentives in order to decrease the use of food crops for biofuel production and instead encourage the use of agricultural residues, such as straws, manures, and slurries (RED II, 2018).

Rice (*Oryza sativa* L.) is one of the most important staple food crops in the world, feeding more than half of the world's population (Tagliapietra et al., 2024), representing the third most important cereal crop in the world (OECD, 2018). Global rice production is estimated to reach 567 million tons by 2030 (Mohidem et al., 2022), while each kg of harvested rice generates 0.7 to 1.5 kg of straw (Grisolia et al., 2022). However, rice is a major contributor to global methane emissions and other greenhouse gases (GHGs) (IPCC, 2022). Specifically, rice cultivation is estimated to account for 10% of the global agricultural GHGs (Maraseni et al., 2018).

Another significant source of GHGs is livestock farming. In Europe, approximately half of the meat production is attributed to swine farming (Akamati et al., 2022). However, intensive swine farming is placing a considerable strain on the environment due to the significant amounts of feed needed, the improper management of wastes, and the high water and energy required (Costantini et al., 2020; Giraldi-Díaz et al., 2021). As a result, swine production is a major source of GHGs, such as methane (CH_4) and carbon dioxide (CO_2), along with other pollutants, including ammonia (NH_4) and hydrogen sulfide (H_2S) (Andretta et al., 2021; Li et al., 2021; Samarin et al., 2021).

Moreover, the European Union (EU) has approximately 76 million head of cattle and ranks as the world's third-largest beef and veal producer (Vinci, 2022). Cattle farming is considered the greatest source of agricultural GHGs, stemming from enteric fermentation and manure land application (Rivera and Chará, 2021; Korres et al., 2023).

Anaerobic digestion (AD) is an effective approach to treat agricultural residues. Among the four stages of AD, i.e., hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Dianat et. al., 2024), the latter is considered to be the rate-limiting step for readily degradable substrates like animal manure, due to the slow growth rate and the sensitivity of the methanogenic archaea to environmental changes (Wang et al., 2023). In contrast, hydrolysis is the rate-limiting step for lignocellulosic biomass (Agregán et al., 2022). Therefore, different methods of overcoming the recalcitrance of plant biomass have been examined, such as physical (Qi et al., 2023; Shaw et al., 2024), biological (Chhetri et al., 2022), and chemical (Xin et al., 2019).

Anaerobic digestion can be characterized as mono-digestion when a single substrate is used and as co-digestion when multiple substrates are utilized (Karki et al., 2021). Rice straw (RS) is a difficult-to-biodegrade substrate, rich in carbon, with a high C:N ratio. In contrast, livestock slurries are easily biodegradable, but highly rich in nitrogen and markedly low in carbon. Consequently, RS and livestock slurries are poor substrates for mono-digestion. RS, due to its slow hydrolysis rate and insufficient nitrogen content for microbial growth, while the high nitrogen content of livestock slurries can lead to the accumulation of toxic levels of ammonia in the digester from excessive protein degradation (Shen et al., 2018). Co-digesting RS with animal slurries secures a balanced C:N ratio, improves biodegradation, prevents ammonia inhibition, and enhances the diversity of the microbial community (Saha et al., 2025). Various nitrogen-rich co-substrates have been successfully combined with RS in AD systems, including cattle (Yin et al., 2023), swine and chicken manure (Mei et al., 2016; Tian et al., 2023), human feces (Adjama et al., 2022), and sewage sludge (Qi et al., 2023).

Despite the clear advantages of co-digestion, biogas production from agricultural residues remains underdeveloped. Therefore, the current study presents a comprehensive analysis aiming to identify the optimum pretreatment methodology for rice straw degradation, coupled with the optimum co-digestion ratio and slurry for maximum methane production. Furthermore, a technoeconomic analysis was employed to assess the feasibility of establishing a biogas power plant co-digesting pretreated rice straw and livestock slurry. Our analysis incorporated costs for pretreatment and highlighted the economic benefits from selling digestate, which are seldom used in technoeconomic analyses.

2. Materials and Methods

2.1 Feedstock

Baled rice straw was collected from a rice paddy in Kalochori, Greece (40°3a7'00.09"N, 22°49'51.93"E). The bale was provided by a local rice farmer who bales rice straw after harvesting for animal bedding and feed (Figure 1).

Figure 1. Rice biomass that was baled and transported from the Kalochori area to the facilities of ELGO-DIMITRA in Thermi, Thessaloniki.

RS feedstock was chopped manually into pieces 2-3 cm long or milled in a laboratory mill to pieces of 0.5 mm (Retsch, model ZM 1000, Germany) (Figure 2).

(a) (b)

Figure 2. Processing of rice straw: a) manually to a length of 2-3 cm (left), b) with a laboratory mill to 0.5 mm pieces (right).

Swine slurry, cattle slurry, and inoculum were procured from a commercial biogas plant in Thessaloniki, Greece (Biogas Lagada S.A.) (Figure 3).

Figure 3. Procurement of effluent (digestate) from the facilities of the 'Biogas Lagada' biogas powerplant.

Once cattle and swine slurry were sieved with a 1 mm mesh to remove foreign materials or large pieces of organic matter, they were transferred into 10 L plastic containers and retained in the freezer (-20 °C) until used in the co-digestion experiments. The inoculum in this study was fresh digestate from the same biogas power plant. After acquisition, it was placed in an incubator (Nabertherm® LH 60/14, Nabertherm, Lilienthal, Germany) for 10 days at 37 °C to ensure the degradation of any remaining readily available organic matter (Table 1).

Table 1. Chemical properties of the rice straw, swine, and cattle slurry used in the current experiment

	Rice straw	Swine slurry	Cattle slurry	Units
Moisture	6.75	92.47	91.75	%
Crude Ash	12.18	3.19	4.65	%
Loss on Ignition	87.82	96.81	95.35	%
VS*	81.07	4.35	4.10	%
Crude Fats	1.19	0.46	0.25	%
Fats in DM*	1.28	6.14	2.99	%
Fats in VS*	1.47	10.63	6.02	% VS*
Crude Proteins	2.94	3.36	2.12	%
Proteins in DM*	3.15	44.57	25.70	%
Proteins in VS*	3.63	77.15	51.71	% VS*

Crude Fibers	32.12	0.51	2.05	% FM*
Fibers in DM*	34.45	6.77	24.81	% DM*
Fibers in VS*	39.62	11.72	23.42	% VS*
NFE*	48.06	0.22	2.36	% DM*
NFE* in VS*	55.28	0.38	16.34	% VS*
pH	8.2	7.9	7.4	-
TS*	93.25	7.53	8.25	%

*FM: Fresh Matter, DM: Dry Matter, VS: Volatile Solids, NFE = Nitrogen free Extracts, TS=Total Solids

2.2 Pretreatment experiments

The thermal pretreatment experiments were conducted following the methodology of Momayez et al. (2017), who used heat and the effluent of a biogas power plant to enhance biomass solubilization. Specifically, rice straw and 16.5 mL of heat-sterilized inoculum were placed in glass reactors with a total volume of 350 mL and a working volume of 100 mL, while the organic loading rate (OLR) was 2 gVS L^{-1}. After adding the mixture, the reactors were sealed with butyl rubber septa and aluminum caps and placed in an incubator (Nabertherm® LH 60/14, Germany) at 130, 160, or 190 °C for 30 or 60 minutes (Figure 4 and Table 2).

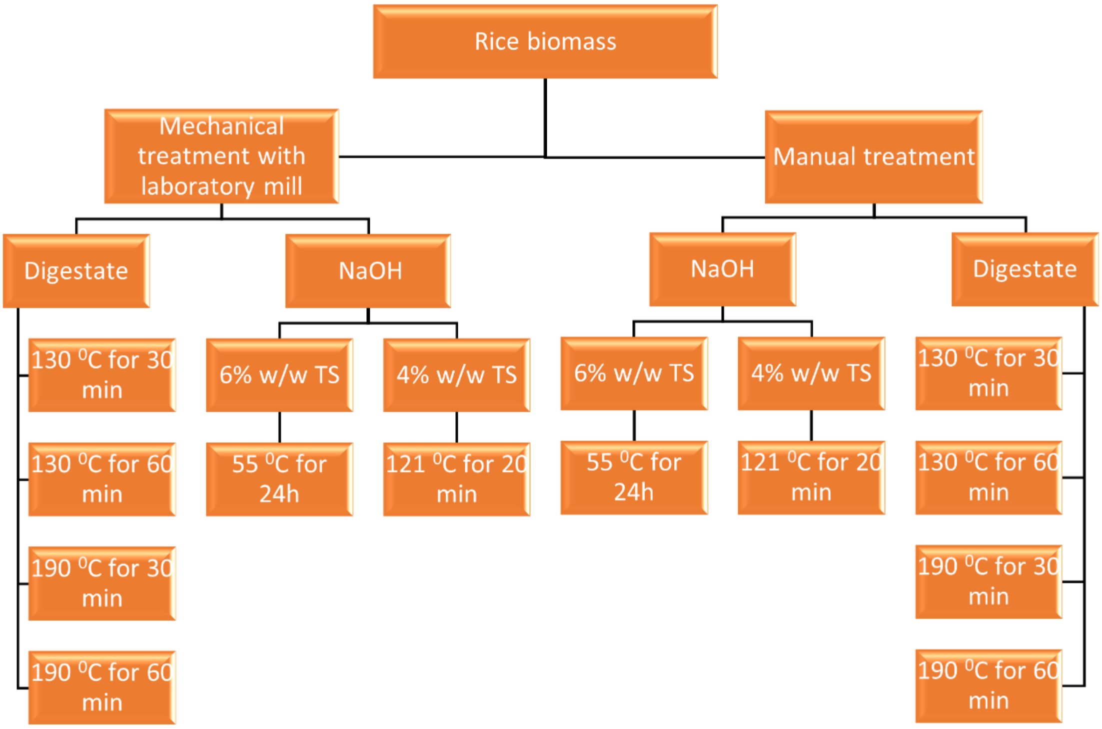

Figure 4. Schematic representation of the different applications for determining the optimal pretreatment method for enhancing rice biomass biodegradation.

Table 2. Conditions applied for the rice straw pretreatment experiment

Rice Straw	
1	Untreated (Rice straw manually chopped 2-3 cm in length) (Control)
2	Mechanical (Rice straw ground by a laboratory mill into pieces of 0.5 mm)
Thermal	
3	Untreated + D*, 130 °C for 30 min (TH1)
4	Untreated + D*, 130 °C for 60 min (TH3)
5	Untreated + D*, 190 °C for 30 min (TH5)
6	Untreated + D*, 190 °C for 60 min (TH7)
7	Mechanical + D*, 130 °C for 30 min (TH2)
8	Mechanical + D*, 130 °C for 60 min (TH4)
9	Mechanical + D*, 190 °C for 30 min (TH6)
10	Mechanical + D*, 190 °C for 60 min (TH8)
Thermochemical	
11	Untreated + NaOH 6% w/w TS* at 55 °C for 24 h (THEC1)
12	Untreated + NaOH 4% w/w TS* at 121 °C for 20 min (THEC3)
13	Mechanical + NaOH 6% w/w TS* at 55 °C for 24 h (THEC2)
14	Mechanical + NaOH 4% w/w TS* at 121 °C for 20 min (THEC4)

*D: Digestate, TS: Total Solids

After pretreatment, reactors were cooled to ambient temperature, pH was adjusted to 7 ± 0.2 (using 1 M HCl) for optimum AD conditions, inoculated at a 2:1 inoculum-to-substrate ratio, received N_2 for 2 minutes to ensure anaerobic conditions, and sealed again. Incubation occurred at mesophilic conditions (37 °C) until biogas production ceased (pressure inside the reactors remained stable for 3 consecutive days).

Samples were manually stirred daily to ensure sufficient contact between the microbes and the substrate. Daily monitoring included pressure measurement inside the reactors using a digital manometer (PCE-005®, PCE Holding GmbH, Germany) (Figure 5).

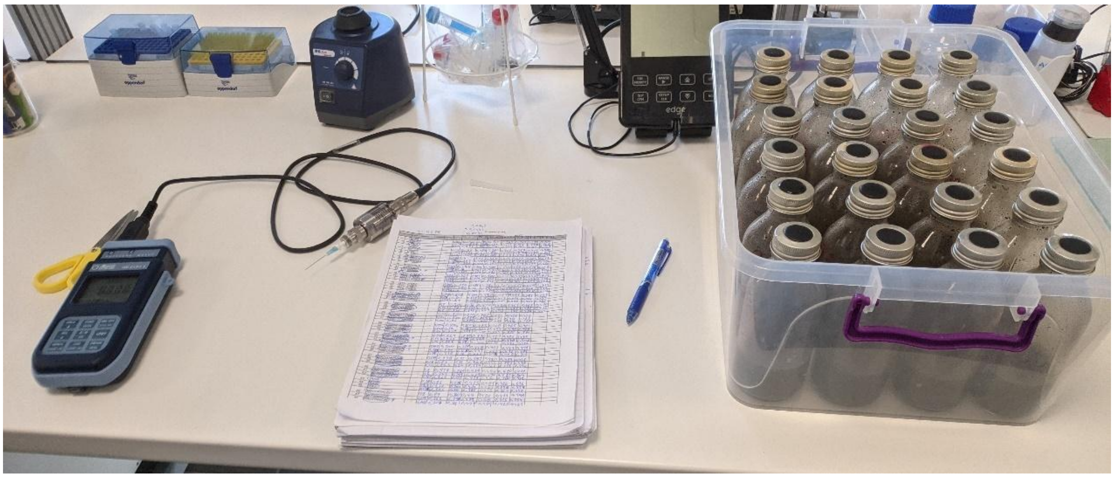

Figure 5. Glass reactors on the right and a digital manometer on the left were used to measure the pressure inside the glass reactors.

Biogas methane content was estimated by gas chromatography (GC-TCD detector, Shimadzu, Japan) (Figure 6).

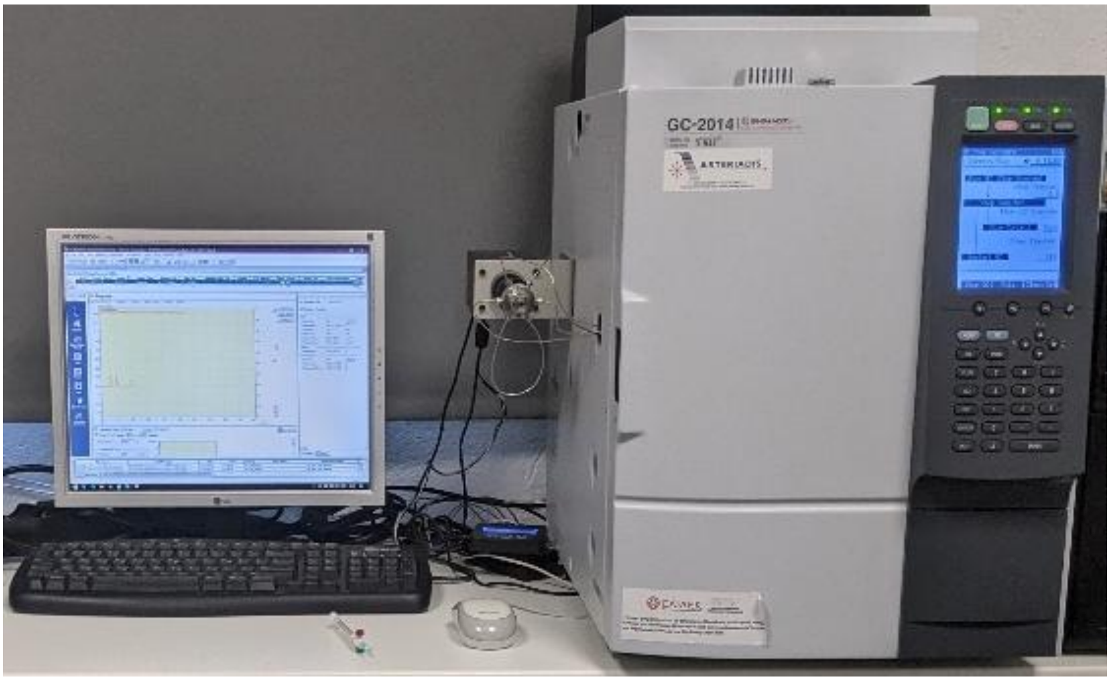

Figure 6. The Gas Chromatograph used for measuring the percentage of methane in the biogas mixture.

The thermochemical experiments were based on the study of Tsapekos et al. (2016). Following their study, rice straw (OLR = 2 gVS L^{-1}) was treated with 1 M NaOH at two concentrations (4% or 6% w/w total solids) and two temperatures (55 °C or 121 °C). Samples with 6% NaOH were incubated for 24 h, while samples receiving 4% NaOH were treated for 20 min. (Figures 4 and 7).

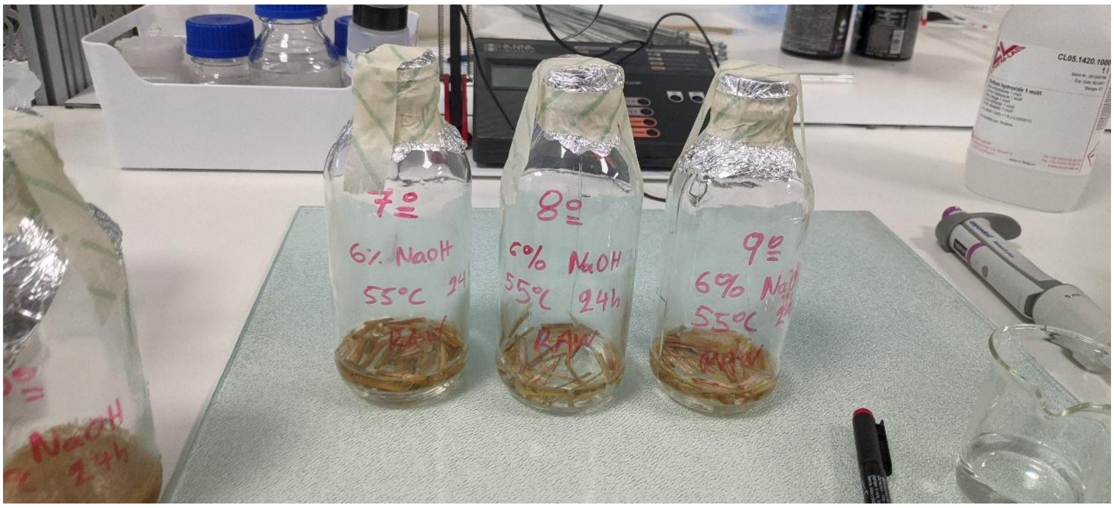

Figure 7. Glass reactors containing rice biomass (0.26 g) and NaOH for thermochemical pretreatment of rice biomass.

After pretreatment, the methodology followed was identical to the thermal pretreatments. All experiments were performed in triplicate, while manually chopped rice straw (2-3 cm pieces) served as the control for all pretreatment experiments.

The efficacy of the pretreatment methods in terms of methane productivity was estimated according to the Biochemical Methane Potential (BMP) protocol of Angelidaki et al. (2009), as referenced in section 2.5.

2.3 Co-digestion experiments

The co-digestion experiments were conducted using identical methodology, reactor specifications, inoculation ratios, and monitoring protocols, as described above. Incubation was conducted at mesophilic conditions (37 °C) until biogas production ceased, i.e., pressure inside the reactors remained stable for 3 consecutive days. The duration of the experiments was approximately 25 days. Process efficiency was evaluated using the BMP protocol (Angelidaki et al., 2009), while all experiments were replicated thrice.

During the co-digestion experiments, five ratios of slurry (cattle or swine) combined with pretreated rice straw (50:50, 60:40, 70:30, 80:20, and 90:10), on a volatile solids basis, were evaluated. Raw slurry (100:0) was used as the control, while the OLR was maintained at 2 gVS L^{-1} (Figures 8, 9, 10).

Figure 8. The glass reactors from the co-digestion experiments placed in a growth chamber (incubator) under mesophilic conditions (37 °C).

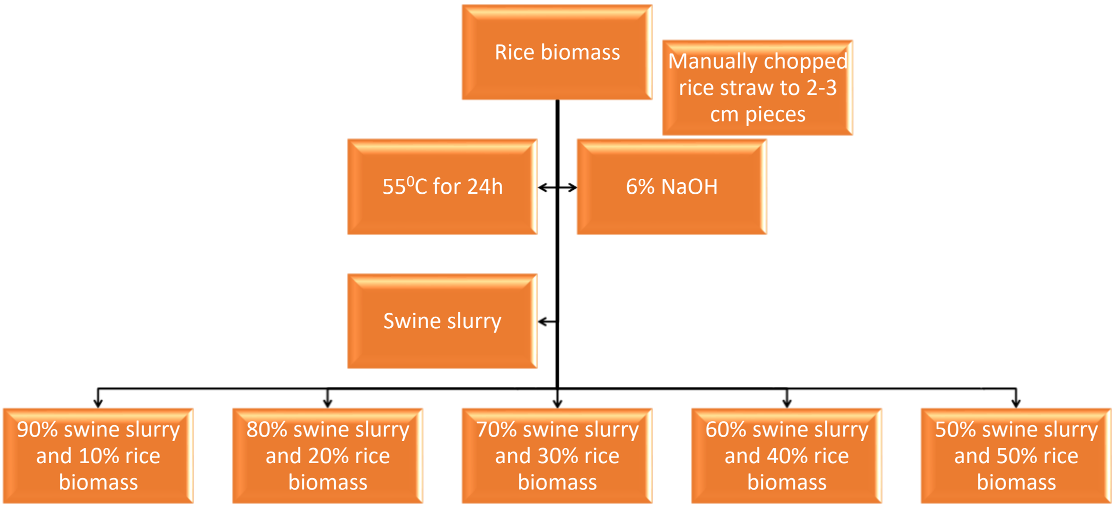

Figure 9. Schematic representation of the different applications for determining the optimal ratio of swine slurry and pretreated rice biomass in the co-digestion experiments.

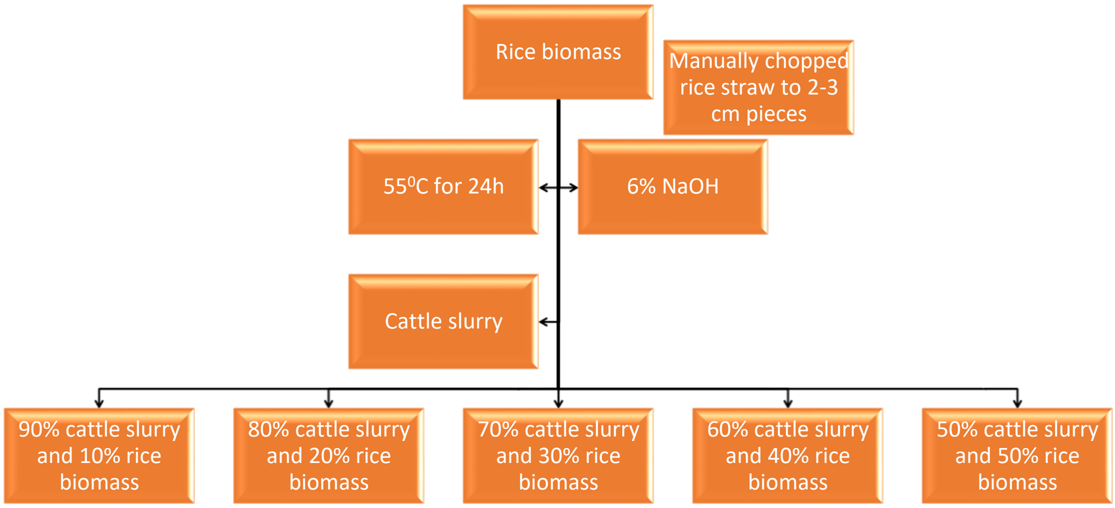

Figure 10. Schematic representation of the different applications for determining the optimal ratio of cattle slurry and rice biomass in the co-digestion experiments.

2.4 Theoretical methane yield

The Theoretical Methane Yield (TMY) was assessed based on the equation of Baserga (1998) and expressed in mL CH_4 gVS^{-1}. According to equations 1 and 2, the TMY is estimated from the percentage of proteins (CP), fats (CF), fibers (CFi), and nitrogen-free extracts (NFE) in the VS. Particularly, equation 1 was used to calculate the Theoretical Biogas Yield (TBY), equation 2 the TMY, and equation 3 the percentage of methane in the biogas (TMY (%)).

$$\text{TBY}\ [\text{mL gVS}^{-1}] = \frac{[(0.57 \text{ x dCP x CP})+(0.8 \text{ x dCF x CF})+(0.64 \text{ x dCFi x CFi})+(0.9 \text{ x dNFE x NFE})]}{100} \quad \text{(Eq. 1)}$$

$$\text{TMY}\ [\text{mL gVS}^{-1}] = \frac{[(700 \text{ x dCP x CP x dfCP})+(1250 \text{ x dCF x CF x dfCF})+(790 \text{ x dCFi x CFi x dfCFi})+(790 \text{ x dNFE x NFE x dfNFE})]}{100}$$

(Eq. 2)

$$\text{TMY (\%)} = \left(\frac{\text{TMY}}{\text{TBY}}\right) \text{x } 100 \text{ (Eq. 3)}$$

where TBY = Theoretical Biogas Yield, TMY = Theoretical Methane Yield, TMY (%) = Percentage of Methane in the biogas

Gas Yield Conversion Factors: dCP = 700, dCF = 1250, dCFi = 790, dNFE = 790

Digestibility Factors: dfCP = 0.71, dfCF = 0.67, dfCFi = 0.52, dfNFE = 0.52

2.5 Analytical methods

Moisture content, Total Solids (TS), pH, and Volatile Solids (VS) were estimated according to standard methods (APHA, 2017). Total nitrogen was determined using the Kjeldahl method (BeMiller, 2010). Lipid levels were obtained through solvent extraction of dried, acidified samples in a Soxhlet apparatus using petroleum ether (O'Keefe and Pike, 2010). Crude fiber content was measured by boiling samples in sulfuric acid and sodium hydroxide solutions, drying, weighing, and incinerating the remaining solids. The loss of weight resulting from ashing corresponds to the crude fiber present in the test sample (Commission Regulation (EC) No 152/2009).

Finally, methane concentration in the biogas samples was analyzed by gas chromatography following the method described by Mitraka et al. (2022).

2.6 Techno-economic analysis

The thermal energy required to heat the feedstock was estimated based on the equation of Kavitha et al. (2015), Eq. 4.

$Q = m \times c \times \Delta T$ (Eq. 4)

Where: m = mass of feedstock and c = specific heat capacity.

We assumed that the specific heat capacity of the feedstock is the same as water = 4.18 kJ/(kg·°C).

ΔT= temperature difference between the temperature required in the digester and the atmospheric temperature.

The Payback Period (PP) is the time required to generate the initial cost of the investment and is calculated using Eq. 5.

$PP = \frac{Initial\ Investment}{Annual\ Revenue}$ (Eq. 5)

Return on Investment (ROI) measures the profitability of an investment expressed as a percentage. ROI can be positive or negative. If it is negative, the investment is not profitable, while if it is positive, the investment is generating profit. It was calculated following Eq. 6.

$ROI = \frac{Annual\ Profit}{Cost\ of\ Investment} \times 100$ (Eq. 6)

Profit Margin (PM) is the ratio of net profit divided by its revenue, expressed as a percentage. PM is revealing the amount of money by which an investment exceeds its costs. It was assessed with the following Eq. 7.

$PM = \frac{Annual\ Profit}{Annual\ Revenue} \times 100$ (Eq. 7)

2.7 Statistical analyses

One-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference test (LSD, $p < 0.05$) was conducted for the determination of significant differences between treatments applied in BMP experiments. ANOVA was carried out using the MSTAT-C ver. 1.41 (Michigan State University, East Lansing, MI, USA) statistical program.

3. Results

3.1 Pretreatment experiments

Significant differences between the various experimental conditions were detected (Figure 11). More specifically, the four thermochemical pretreatments exhibited the highest increases in methane yield compared to the control, while no differences were observed amongst them (i.e., methane yields ranged from 315 to 321 mL CH_4 gVS^{-1} and were 13% to 15% higher than the control).

Thermal pretreatments TH4, TH2, TH8, and TH5 generated 294, 290, 286, and 285 mL CH_4 gVS^{-1}, respectively, and increased methane production by 2% up to 5%, compared to the control. In contrast, pretreatments TH1, TH3, and TH6 exhibited reductions in methane production compared to the control, ranging from 1% to 6%, while TH7 showed the highest reduction, equal to 15% (Figure 11).

Furthermore, the thermochemical pretreatments exhibited the lowest differences with the theoretical methane yield of the control (TMY), ranging from -2.4% to -4.2%. Conversely, the thermal samples exhibited the highest differences, ranging from -11.8% to -28% (Figure 11).

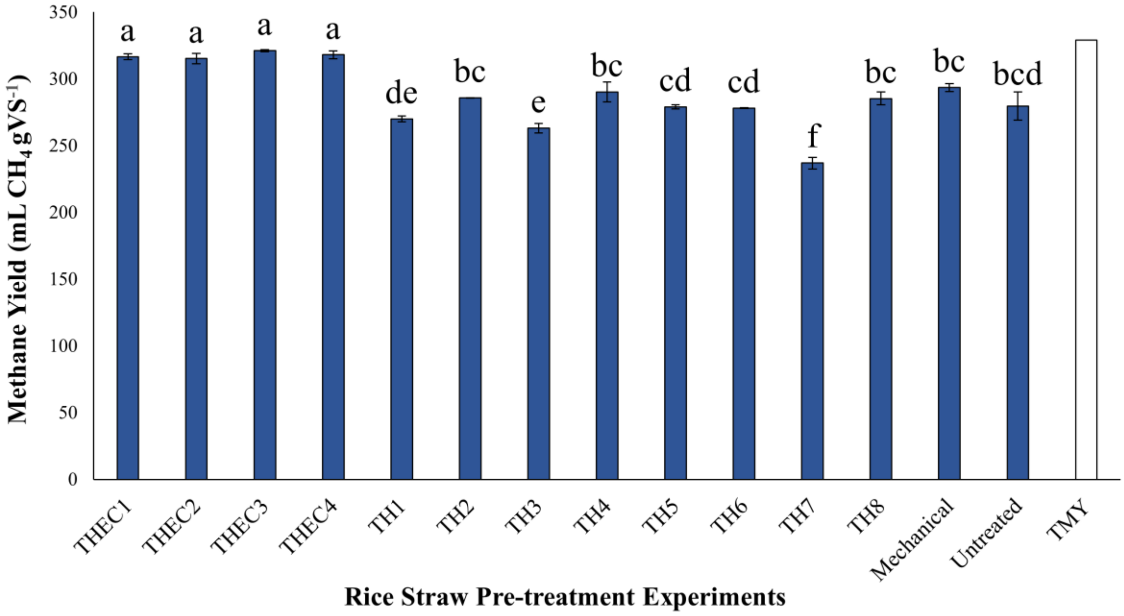

Figure 11. Bar chart of statistically significant differences between the pretreated samples and the untreated rice straw's Theoretical Methane Yield (TMY). The white bar represents the TMY of untreated rice straw. Different letters indicate differences between samples at a = 0.05. TMY=Theoretical Methane Yield of untreated rice straw, THEC1 = Thermochemical pretreatment of Untreated RS with 6% NaOH, at 55 °C, for 24 h, THEC2 = Thermochemical pretreatment of Mechanically treated RS with 6% NaOH, at 55 °C, for 24 h, THEC3 = Thermochemical pretreatment of Untreated RS with 4% NaOH, at 121 °C, for 20 min, THEC4 = Thermochemical pretreatment of Mechanically treated RS with 4% NaOH, at 121 °C, for 20 min, TH1 = Thermal pretreatment of Untreated RS with D, at 130 °C, for 30 min, TH2 = Thermal pretreatment of Mechanically treated RS with D, at 130 °C, for 30 min, TH3 = Thermal pretreatment of Untreated RS with D, at 130 °C, for 60 min, TH4 = Thermal pretreatment of Mechanically treated RS with D, at 130 °C, for 60 min, TH5 = Thermal pretreatment of Untreated RS with D, at 190 °C, for 30 min, TH6 = Thermal pretreatment of Mechanically treated RS with D, at 190 °C, for 30 min, TH7 = Thermal pretreatment of Untreated RS with D, at 190 °C, for 60 min, TH8 = Thermal pretreatment of Mechanically treated RS with D, at 190 °C, for 60 min, Mechanical = Rice straw mechanically grinded by a laboratory mill in pieces of 0.5 mm, Untreated = Rice straw manually chopped 2-3 cm in length and served as the control.

Although all four thermochemical pretreatment methods exhibited the highest methane yields and did not show significant differences among them, only one could be used for the continuation of the experimental process. Subsequently, THEC1 was chosen as it was the least energy-demanding.

3.2 Pretreated rice straw and cattle slurry co-digestion batch experiments

According to our results, increases in methane yield were linked to increases in p-RS. Specifically, 50CS exhibited the highest methane yield, producing 14% more methane than the control (100CS), while 60CS and 70CS followed with modest increases of 2% and 5%, respectively. Conversely, the methane yield of 90CS was 11% lower than the control (Figure 12).

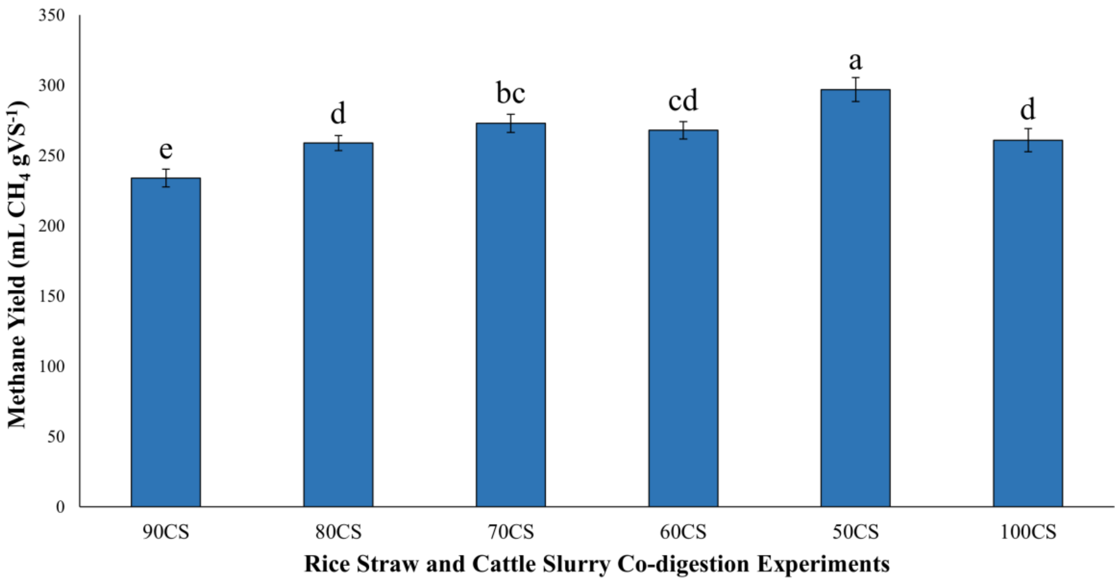

Figure 12. Bar chart of statistically significant differences between the co-digested samples. Different letters indicate differences between samples at a = 0.05. 100 CS = 100% Cattle Slurry, 90CS = 90% Cattle Slurry and 10% p-RS, 80CS = 80% Cattle Slurry and 20% p-RS, 70CS = 70% Cattle Slurry and 30% p-RS, 60CS = 60% Cattle Slurry and 40% p-RS, 50CS = 50% Cattle Slurry and 50% p-RS.

Moreover, 50CS exhibited the lowest percentage difference with the theoretical methane yield of the control (TMY 100CS), producing 3.9% less methane than TMY 100CS. 70CS and 60CS followed with 11.7% and 13.3% less methane, respectively, while 90CS exhibited the highest percentage difference (24.3% less) (Figure 13). Notably, most of the examined samples outperformed their theoretical counterparts. Specifically, all of the examined samples outperformed their theoretical counterparts by 7.2% to 13.8%, except 90CS and 100CS, which produced 1.3% and 15.5% less, respectively (Figure 13).

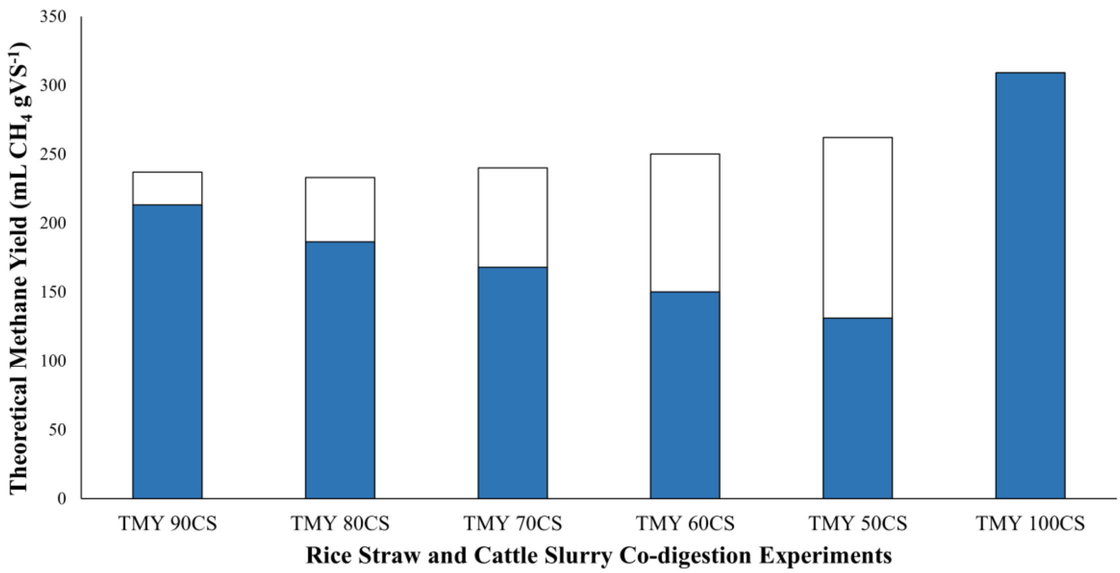

Figure 13. Bar chart of the Theoretical Methane Yield of the co-digested samples. The two colors of the bars represent the ratio of Cattle Slurry (CS) and pretreated Rice Straw (p-RS) in the co-digestion mixture. Blue color represents CS, and white color p-RS. TMY 100CS = Theoretical Methane Yield of 100% Cattle Slurry, TMY 90CS = Theoretical Methane Yield of 90% Cattle Slurry and 10% p-RS, TMY 80CS = Theoretical Methane Yield of 80% Cattle Slurry and 20% p-RS, TMY 70CS = Theoretical Methane Yield of 70% Cattle Slurry and 30% p-RS, TMY 60CS = Theoretical Methane Yield of 60% Cattle Slurry and 40% p-RS, TMY 50CS = Theoretical Methane Yield of 50% Cattle Slurry and 50% p-RS.

3.3 Pretreated rice straw and swine slurry co-digestion batch experiments

The general trend was that as the percentage of swine slurry increased, methane yield decreased (Figure 14). In particular, 50SS presented the highest methane yield (315 mL CH_4 gVS^{-1}), exhibiting a 59% increase compared to the control (100SS). 60SS and 70SS followed with 44% and 41% increases, respectively. Conversely, 80SS and 90SS demonstrated the lowest increments in methane yield, at 33% and 30%, achieving 263 and 258 mL CH_4 gVS^{-1}, respectively (Figure 14).

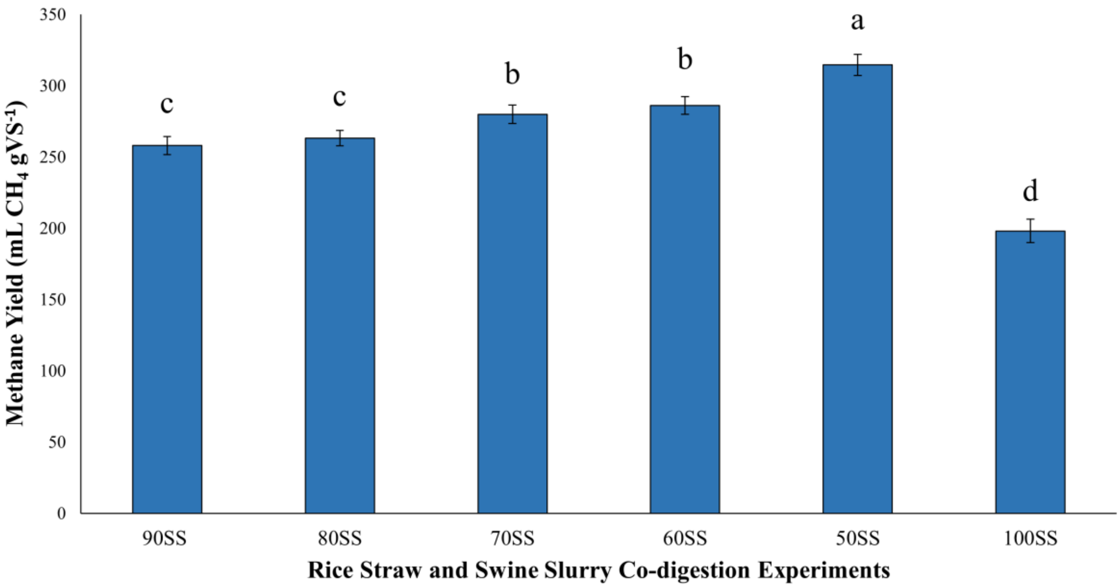

Figure 14. Bar chart of statistically significant differences between the co-digested samples. Different letters indicate differences between samples at a = 0.05. 100 CS = 100% Swine Slurry, 90CS = 90% Swine Slurry and 10% p-RS, 80CS = 80% Swine Slurry and 20% p-RS, 70CS = 70% Swine Slurry and 30% p-RS, 60CS = 60% Swine Slurry and 40% p-RS, 50CS = 50% Swine Slurry and 50% p-RS.

In addition, 50SS demonstrated the lowest percentage difference (-2.2%) with the theoretical methane yield of the control (TMY 100SS). Conversely, 90SS presented the highest percentage difference, equal to -19.8%. Crucially, almost all of the examined treatments outperformed their corresponding TMYs. Particularly, all of the examined samples exceeded their TMYs by 2% to 13.2%, with the sole exception of 90SS, producing 2% less (Figure 15).

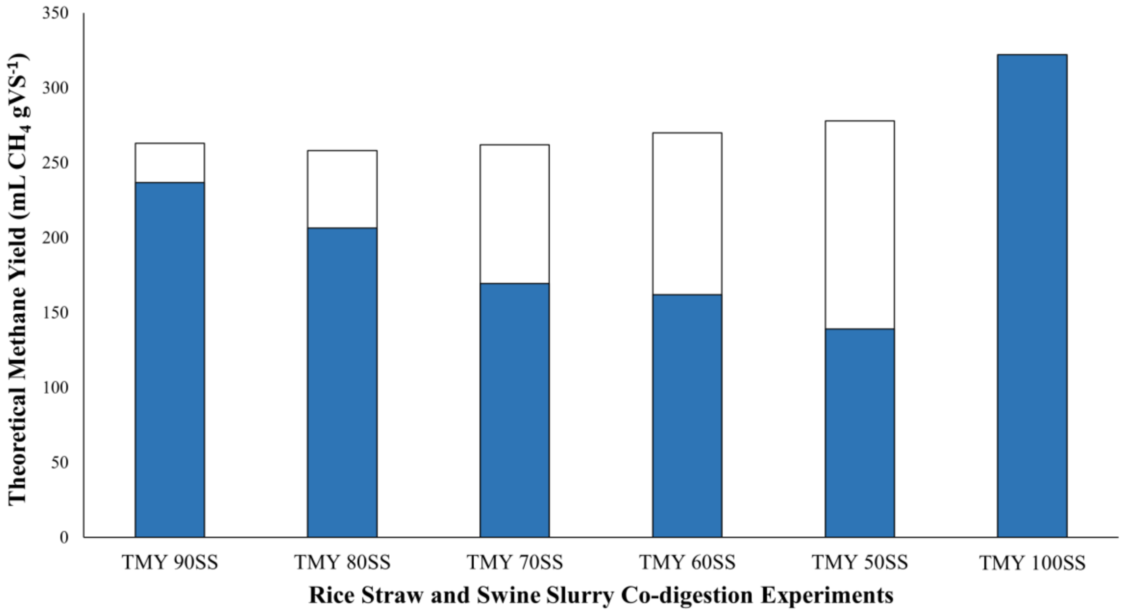

Figure 15. Bar chart of the Theoretical Methane Yield of the co-digested sample. The two colors of the bars represent the ratio of Swine Slurry (SS) and pretreated Rice Straw (p-RS) in the co-digestion mixture. Blue color represents SS, and white color p-RS. TMY 100CS = Theoretical Methane Yield of 100% Swine Slurry, TMY 90CS = Theoretical Methane Yield of 90% Swine Slurry and 10% p-RS, TMY 80CS = Theoretical Methane Yield of 80% Swine Slurry and 20% p-RS, TMY 70CS = Theoretical Methane Yield of 70% Swine Slurry and 30% p-RS, TMY 60CS = Theoretical Methane Yield of 60% Swine Slurry and 40% p-RS, TMY 50CS = Theoretical Methane Yield of 50% Swine Slurry and 50% p-RS.

3.4 Techno-economic analysis

Concerning the techno-economic analysis, we assumed that the biogas power plant's feedstock would comprise 70% SS and 30% p-RS (70SS in Figure 14). Although 50SS was the optimum co-digestion mixture (Figure 14), using such a high percentage of rice straw in the feedstock could be challenging for the plant, because RS is a seasonal feedstock, expensive to collect and transport, and requires pretreatment (Torregrosa et al., 2021). In contrast, SS is cheaper to transport and readily available year-round. Therefore, 70SS was selected as it produced the second-highest methane yield with the least quantity of rice straw.

The cost for acquiring and installing the combined heat and power (CHP) unit was estimated at €4,500,000 based on the study of Benato and Macor (2019). Moreover, we assumed an electrical efficiency of 40.6% (ηe) and a thermal efficiency of 43.4% (ηt) (Spyridonidis et al., 2020). Consequently, the electric energy produced would be 8,760 MWh year^{-1} and the thermal energy 9,364 MWh year^{-1} (Table 3). Therefore, the combined energy output of the CHP unit would be 84%, while the remaining 16% would be lost during the biogas-to-electricity conversion process.

Table 3. Energy output for a 1 MW Biogas Power Plant

Total Energy Produced
1. Electrical: 8,760 MWh year^{-1}
2. Thermal: 9,364 MWh year^{-1}

The parasitic electricity demand for the CHP engine was assumed at 6.5% of the gross electrical production based on the study of Spyridonidis et al. (2020), while an additional 7% was added for various other parasitic loads (Scholwin and Nelles, 2013) (Table 4). Heat losses were calculated as 20% of the energy required to heat the feedstock. The thermal energy required to pretreat the feedstock and other thermal losses were assumed to be an additional 2.6% of total thermal energy production. In summary, the gross parasitic electricity consumption was calculated as 13.5% of gross electrical output, and the gross parasitic thermal consumption as 11.2% of gross thermal output, including RS grinding and pretreatment.

Table 4. Parasitic energy consumption

Parasitic electrical consumption
1. CHP unit electrical consumption: 569 MWh year^{-1}
2. *Other electrical parasitic loads: 613 MWh year^{-1}
Parasitic thermal consumption
1. Energy required to heat the feedstock: 672 MWh
2. Heat loss from the digester: 135 MWh
3. Feedstock pretreatment and other heat losses: 244 MW
Net energy output
Electrical: 7,578 MWh year^{-1}
Thermal: 8,313 MWh year^{-1}

* Other Electrical Parasitic loads include grinding the rice biomass, pumps, power for the stirrer, lights, etc.

The economic analysis for a biogas power plant treating swine slurry and rice biomass (70% SS and 30% p-RS) is presented in Table 5. Apart from the electrical and thermal energy sales, another revenue source considered was digestate sales, which represent an untapped source of income. According to Jurgutis et al. (2021), the average value of the digestate produced daily from a 1 MW biogas power plant is €1,518. Assuming the plant operates 365 days year^{-1}, the increase in revenue can be as high as €554,070 year^{-1}.

For slurry transportation, we assumed the use of an 18-ton slurry truck (Spyridonidis et al., 2020). Considering a round trip of 40 km at an average speed of 25 km hour^{-1} and operating on an 8-hour daily schedule for 6 days a week over 50 weeks annually, a single tanker can transport 21,600 tons year^{-1}. Consequently, to transport the required swine slurry (23,841 tons year^{-1}), two slurry tankers would be needed.

According to Torregrosa et al. (2021), the cost of transporting and handling rice straw from the paddy fields to a stocking area 25 km away is between €28.1 and €51 per ton. Therefore, a mean value of €39 ton^{-1} was assumed for this study. Additionally, an estimated 571.64 tons of NaOH y^{-1} would be needed for rice biomass pretreatment, with an approximate cost of €403.28 ton^{-1} (Chemanalyst, 2024), equal to €230,531 year^{-1}.

The annual operational and maintenance (O&M) cost was estimated at €147,500 year^{-1} based on the mean values reported by Benato and Macor (2019).

The plant would treat 23,841 tons of swine slurry and 10,217 tons of rice biomass (in total, 34,058 tons y^{-1}). Each pig produces a mean of 2.22 tons of slurry per year (Schiavon et al., 2010); therefore, to obtain the required 23,841 tons year^{-1}, approximately 10,739 pigs are needed. In the prefecture of Central Macedonia, there are 124,013 farmed pigs, according to the 2020 national livestock survey (ELSTAT, 2022a). Hence, approximately 10% of all the pigs in Central Macedonia would be needed.

According to Matías et al. (2019), the mean rice straw yield per hectare is 9.7 tons. Therefore, 1,054 hectares are required per annum. As the prefecture of Central Macedonia has more than 20,000 hectares (ELSTAT, 2022b), constructing the power plant there would be advantageous.

Currently, Greece has a fixed price of €225 per MWh (Legislation 149, 2016) for electricity produced by anaerobic digestion power plants, while the price for thermal energy, according to Herbes et al. (2018), is estimated at €19.1 per MWh.

Based on our analysis, the total annual revenue would be €2,417,898, including the earnings from digestate sales, and at €1,863,828 without digestate sales. The net profit was calculated as €1,509,428 with digestate revenue and €955,358 without. The profit margin (PM) was estimated at 62.43% with the sale of digestate and 51.26% without. Finally, the Payback Period (PP) was calculated at 2.98 and 4.71 years with and without digestate revenue, and the Return on Investment (ROI) at 33.54% and 21.23% year^{-1}, with and without digestate revenue, respectively (Table 5).

Table 5. Economic analysis comparing profits with and without the revenue from digestate

Revenue with digestate	Revenue without digestate
Electricity: €1,705,050 year^{-1} Heat: €158,778 year^{-1} Digestate: €554,070 year^{-1}	Electricity: €1,705,050 year^{-1} Heat: €158,778 year^{-1} Digestate: 0
Annual revenue: €2,417,898	Annual revenue: €1,863,828
Liabilities	Liabilities
*O&M: €147,500 year^{-1}	*O&M: €147,500 year^{-1}

Rice straw transportation: €404,082 $year^{-1}$ Swine slurry transportation: €126,357 $year^{-1}$ NaOH: €230,531 $year^{-1}$ Total: €908,470	Rice straw transportation: €404,082 $year^{-1}$ Swine slurry transportation: €126,357 $year^{-1}$ NaOH: €230,531 $year^{-1}$ Total: €908,470
Net annual profit: €1,509,428	Net annual profit: €955,358
Financial metrics	Financial metrics
*PP: 2.98 years *ROI: 33.54% $year^{-1}$ *PM: 62.43%	*PP: 4.71 years *ROI: 21.23% $year^{-1}$ *PM: 51.26%

*O&M = Operational and maintenance cost, PP = Payback period, ROI = Return on investment, PM = Profit margin

4. Discussion

4.1 Rice straw pretreatment

Mechanical size reduction of anaerobic feedstock is a simple and practical pretreatment approach. It increases accessible surface area and porosity, and reduces cellulose crystallinity and degree of polymerization by decreasing particle size (Di Domenico et al., 2025). Jomnonkhaow et al. (2021) reported that the reduction in particle size increases biodegradability by releasing cell components and creating new surfaces that microbes can access. Mothe et al. (2024) found that size reduction of anaerobic feedstock enhanced bacterial activity, morphology, and dissolution capabilities. However, our milling of rice straw to 0.5 mm particles provided only a 5% increase in methane yield, compared to rice straw chopped to 2-3 cm pieces. Similar results were found by Tsapekos et al. (2016), who reported insignificant improvements from mechanically treating lignocellulosic biomass. The results of our experiments indicate that enhancing biomass degradability through size reduction is limited, and other pretreatments are needed to meaningfully increase rice straw biodegradability.

In contrast to physical pretreatments, chemical methods such as alkaline hydrolysis can significantly enhance biomass digestibility by delignifying and disrupting cellulose crystallinity (Shakeel et al., 2024). Alkaline pretreatment works by saponifying and cleaving lignin-carbohydrate ester bonds, increasing porosity and surface area, swelling the structure, and reducing cellulose crystallinity and polymerization (Shakeel et al., 2022). Moreover, the use of moderate temperatures (55 °C) during pretreatment prevents the formation of recalcitrant compounds such as furfural and 5-HMF that occur during high-temperature alkaline or acidic pretreatment (Ahmed et al., 2022; Balasundaram et al., 2022). These recalcitrant compounds, formed through Maillard reactions, inhibit methanogenic activity and reduce biogas yields.

Other researchers have investigated acid pretreatment of recalcitrant substrates such as rice straw. According to Woźniak et al. (2025), pretreating lignocellulosic biomasses with strong acids at mild temperatures has a high saccharification efficiency; however, acid neutralization and recovery are costly. In contrast, treatment with dilute acid is cheaper; however, it is less efficient and requires high temperatures, which can lead to the production of toxic by-products necessitating removal prior to AD.

Prior studies have demonstrated enhanced biogas yields from lignocellulosic biomasses preheated to temperatures below 140 °C (Rajput et al., 2021; Luo et al., 2019). Conversely, the thermal pretreatments in our study did not markedly increase rice straw biogas production, with only 3 out of 8 conditions showing minor increases versus the control. However, the combined effect of the thermal and alkali pretreatment with NaOH showed significantly higher increases in methane yield, signifying that thermal pretreatment alone is inadequate to meaningfully enhance RS degradability. According to Wang et al. (2018), pretreatment at 190 °C severely inhibits methane production due to the formation of toxic substances. In our study, pretreatment at 190 °C presented the lowest methane production, consistent with the findings of Wang et al. (2018).

4.2 Co-digestion of pretreated rice straw with livestock slurries

Cattle and swine manure are effective co-substrates for anaerobic co-digestion with rice straw, due to their high moisture content and nutrient level, facilitating a stable anaerobic digestion process (Muhayodin et al., 2021).

Singh et al. (2024) reported that co-digesting rice straw with cow dung balances the C:N ratio to 20-30, enhancing biogas production. Jugal Sukhesh and Venkateswara Rao (2019) reported that the co-digestion of dairy manure with rice straw improved methane yields by 50%-57% and 9%-10%. In another study performed by Ona and Agog (2022), the optimal ratios for biogas production from the co-digestion of bovine manure and rice straw were 2:1 and 1:1. In our experiments, the 50-50 setup presented the highest methane production, while the 70-30 mixture produced the second highest methane yield, regardless of slurry. These results demonstrate the importance of RS in stabilizing the AD process and increasing methane production, compared to the mono-digestion of livestock wastes.

Zhong et al. (2021) reported enhanced methane yields when rice straw, bio-pretreated with cellulolytic microflora, was anaerobically digested with swine manure. Similar results were found by Tian et al. (2023), reporting significant increases in methane production through the co-digestion of swine manure and rice straw. According to the authors, these increases resulted from a more balanced nutrient feedstock, enhanced microbiota diversity, and synergy.

Co-digestion offers significant advantages over mono-digestion, such as enhanced feedstock buffer capacity, enrichment with trace elements, dilution of toxic substances, and improved moisture content (Kadam et al., 2024). In addition, co-digestion of recalcitrant substrates, such as RS, with livestock wastes alters the structure and dynamics of the microbial community, increases the microbial and enzymatic diversification in the feedstock, and creates synergistic effects, enhancing substrate biodegradability and methane production (Sudiarto et al., 2025).

The superior performance of co-digestion (up to 59% improvement) stems from addressing the primary limitations of mono-digestion. Rice straw mono-digestion suffers from a high C:N ratio and slow biodegradation (Mothe and Polisetty, 2021). Conversely, swine slurry mono-digestion can result in ammonia toxicity due to its high nitrogen content (Yang et al., 2025). Co-digestion creates a balanced nutrient environment and dilutes excess nitrogen, thereby preventing ammonia toxicity (Kadam et al., 2024). Furthermore, the diverse microbial communities in livestock slurries introduce specialized cellulolytic and xylanolytic bacteria, absent in lignocellulosic biomasses, enhancing rice straw biodegradability (Jensen et al., 2021).

4.3 Techno-economic analysis

In our scenario, we assumed that a 1 MW biogas power plant would be constructed in the prefecture of Central Macedonia, Greece. The location was chosen based on the availability of raw materials in the area. The electrical and thermal efficiency (40.6% and 43.4%, respectively) was assumed to be identical to an actual biogas power plant constructed in Xanthi, Greece (Spyridonidis et al., 2020). The combined 84% efficiency is comparable to the 87% efficiency reported by Ciuła et al. (2023) for a biogas power plant treating agricultural residues.

Spyridonidis et al. (2020) reported that a CHP unit consumes 6 to 7% of the produced electrical energy. In our study, total parasitic energy consumption was estimated at 24.7% (13.5% electrical and 11.2% thermal energy). Electrical and especially thermal energy consumption are both considerably high in our study due to rice straw requiring pretreatment. However, this energy consumption is necessary because, without pretreatment, rice straw exhibits significantly lower digestibility and biomethanation potential. Moreover, parasitic energy is difficult to estimate accurately because it is influenced by a multitude of factors, such as feedstock type and pretreatment, heat insulation efficiency, operational conditions, etc. (Knauer et al., 2018; Ahmadi et al., 2023).

The total cost for the biogas power plant, including the CHP unit, was estimated at €4,500,000, based on the mean value of a 1 MW power plant in Italy (Benato and Macor, 2019). In the study of Spyridonidis et al. (2020), the total cost was €2.5 million. However, the facility in their study produced half the electrical power (500 kW) compared to our scenario.

According to Perta et al. (2019), the O&M cost of a biogas power plant ranges between €172,000 and €174,000 per year, slightly higher than in our study (€147,500 $year^{-1}$). Our estimation was based on Benato and Macor (2019), who reported that the annual O&M cost for a 1 MW plant is between €115,000 and €180,000 $year^{-1}$.

According to Pochwatka et al. (2023), the payback period (PP) for a 499 kW biogas plant operating 12 h day^{-1} is approximately 3.41 years, while Perez et al. (2024) claimed that a biogas plant treating sewage sludge operating 24 h day^{-1} has a maximum PP of 2 years. Other researchers have suggested considerably higher PPs, closer to 8.0 years, such as Nitamakwuavan and Rahim (2022) and Aggarangsi et al. (2023).

However, most of these studies have excluded potential revenues from selling digestate. In our research, removing the earnings from digestate would substantially reduce profitability, resulting in a net profit of €955,358 and increasing the PP to 4.7 years. Conversely, if we add the revenue from digestate, net profit increases by 58% to €1,509,428, and PP decreases to 2.98 years. These findings demonstrate the importance of making digestate a marketable product.

In summary, the project appears viable but sensitive to fluctuations in electricity prices, the overall cost of feedstock procurement, and pretreatment. However, we have not considered any potential income from future gate fees or construction subsidies, which would greatly affect profitability and construction costs, respectively.

Moreover, the EU is incentivizing, through legislation, the construction of more biogas units and the use of agricultural residues in AD plants instead of energy crops (RED II, 2018). Additionally, digestate has been included in the exhaustive list of fertilizers, soil conditioners, and nutrients allowed in organic farming in the EU (Commission Delegated Regulation (EU) 2021/1768), while according to the latest Greek legislation, the use of digestate in fields will be subsidized at €110 ha^{-1} (Legislation 1384/190515/2023). These European and national strategies will positively impact the sustainability and profitability of this project and thus make it more appealing to potential investors.

4.4 Scale-up considerations and environmental trade-offs

Upscaling from batch experiments to an industrial scale faces several operational constraints. NaOH consumption (571.64 tons year^{-1}) requires a reliable supply network and storage infrastructure. The 55 °C pretreatment temperature necessitates continuous heating, consuming approximately 244 MWh annually (Kavitha et al., 2015). Unprocessed rice straw has low density (86-114 kg m^{-3}), making transportation, handling, processing, and storage costly and difficult (Van Hung et al., 2020).

In addition, the use of NaOH can significantly increase the sodium concentration in the digestate, making it unusable by farmers. High sodium concentration can inhibit soil hydraulic conductivity, inducing soil hardening and reducing aeration (Lamolinara et al., 2022). Alternative alkaline agents (KOH, $Ca(OH)_2$, ammonia) could mitigate sodium accumulation but require economic reevaluation. Chemical recovery through membrane filtration could reduce NaOH consumption; however, this would lead to increases in capital investment.

Moreover, rice straw is a seasonal feedstock, requiring adequate storage with fire prevention systems. Collection and transportation of straw from fields are time-consuming and require a network of collection centers and a well-regulated supply chain management (Singh et al., 2024). The 40 km collection radius assumes existing road infrastructure, while remote paddies may increase transportation costs.

5. Conclusions

This study demonstrates that thermochemically pretreating rice straw significantly increases RS methane production (up to 15%), while thermal pretreatment offers limited increases up to 5%, compared to untreated RS.

Moreover, co-digesting p-RS with animal slurries increases methane production up to 59% when using swine slurry, and up to 14% when using cattle slurry, compared to the mono-digestion of each slurry. The synergistic effect, the enhanced microbial diversity, and the balanced C:N ratio stemming from the co-digestion of p-RS and livestock slurries led to higher and more efficient substrate biodegradation, resulting in elevated methane yields.

The techno-economic analysis reveals strong commercial viability (2.98 years PP, 33.54% ROI year^{-1}) for a 1 MW biogas power plant in Greece treating p-RS and swine slurry. The plant can process 34,058 tons of waste annually and generate 15,891 MWh year^{-1} of renewable energy. Notably, digestate sales increased net profit by 58%, demonstrating digestate's potential to enhance the biogas power plants' profitability. These results can be used throughout the Mediterranean rice-growing region as a paradigm, demonstrating that sustainable energy production from rice cultivation and livestock residues is economically feasible.

Future research should include a life cycle assessment (LCA) analysis to quantify the full environmental benefits of this integrated system, including greenhouse gas emission reductions and fossil fuel displacement. Furthermore, investigating hydrolysis kinetics and startup time could elucidate the mechanisms enhancing methane production from co-digestion versus mono-digestion, facilitating further process optimization.

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Chapter 4: Effect of gradual and instantaneous organic loading rate increases on methane production during co-digestion of rice straw and swine slurry

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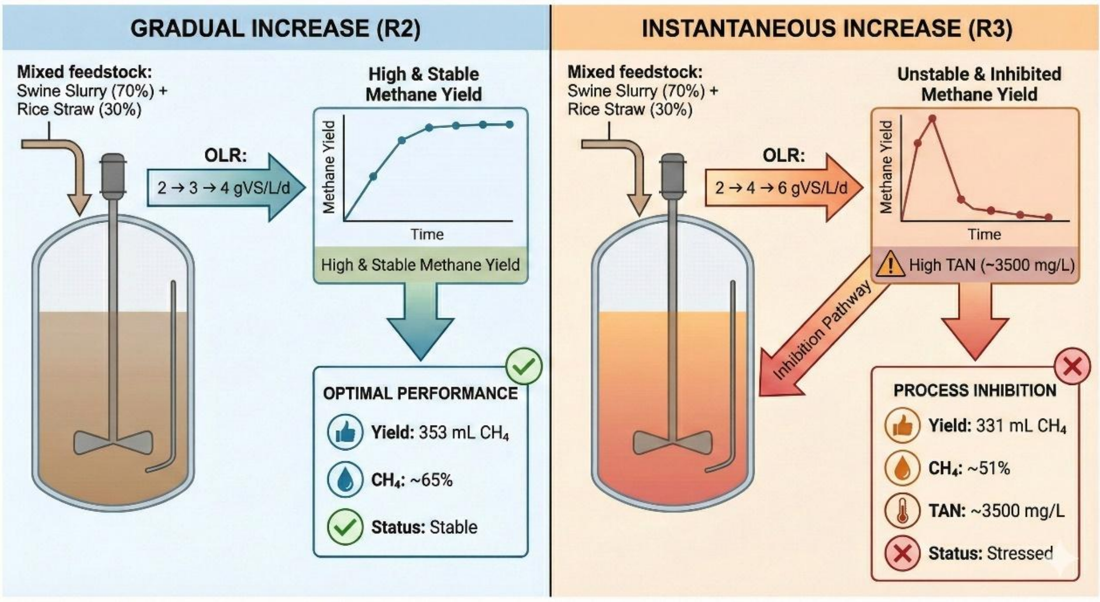

Abstract

Anaerobic digestion generates renewable energy and provides a sustainable method for managing agricultural waste. Nevertheless, feedstock composition and operational parameters can significantly affect process efficiency. In the current study, three mesophilic continuous stirred tank reactors (R1, R2, and R3) were employed to assess the performance of a system co-digesting pretreated rice straw with swine slurry and to evaluate the effects of stepwise (instantaneous) and gradual increases in organic loading rate (OLR). Results demonstrated that the highest methane yield (353 mL CH_4 gVS^{-1}) was achieved at a stable OLR of 2 gVS L^{-1} d^{-1}. Gradual increases in OLR up to 4 gVS L^{-1} d^{-1} caused only transient reductions in methane yield, highlighting the system's capacity for recovery. In contrast, stepwise OLR increases up to 6 gVS L^{-1} d^{-1} significantly suppressed methane production (331 mL CH_4 gVS^{-1}) and reduced biogas methane content (51%). These decreases were closely associated with elevated total ammonia nitrogen (TAN) (~3,500 mg L^{-1}) and free ammonia (FA) (212 mg L^{-1}). A strong negative correlation ($r = -0.826$) was found between TAN and methane yield, while chemical oxygen demand (COD) removal efficiency (65–67%) remained adequate even under stress conditions induced by OLR increases. Overall, gradual increases in OLR proved far more favorable than stepwise ones, as they allowed gradual adaptation of the system and minimized process instability. Furthermore, the co-digestion of swine slurry with pretreated rice straw demonstrated excellent potential as a feedstock combination for anaerobic digestion.

1. Introduction

Anaerobic digestion (AD) is an environmentally friendly process that produces renewable energy while simultaneously treating agricultural residues in a sustainable manner (Sihlangu et al., 2024). Several agricultural and agro-industrial residues are eligible for AD, including crop residues, livestock waste, and by-products from the food industry and the agro-industrial sector (Ngabala and Emmanuel, 2024).

Anaerobic digestion is a complex microbial process that aims to decompose organic matter under anoxic conditions and comprises four stages, namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Zamri et al., 2021). During hydrolysis, complex organic polymers such as carbohydrates, proteins, and lipids are broken down into simpler soluble molecules such as sugars, amino acids and fatty acids. These simpler molecules further degrade to Volatile Fatty Acids (VFAs), including acetic, propionic, and butyric acids, along with alcohol, hydrogen, carbon dioxide, and ammonia, during the second stage of AD called acidogenesis. In the third stage (acetogenesis), acetogenic bacteria convert the non-acetate VFAs and alcohols into additional acetic acid, hydrogen and carbon dioxide (Rezapoor and Rahimpour, 2024). The final stage of methanogenesis involves the methanogenic archaea converting acetic acid, carbon dioxide and hydrogen to biogas, mainly through the acetoclastic and hydrogenotrophic pathways (Kougias and Angelidaki, 2018).

The operational parameters within the digester exert a crucial influence on the efficiency of the anaerobic digestion process and the effective degradation of the organic matter (Jameel et al., 2024). Parameters such as OLR, temperature and pH are key components for enhanced organic matter degradation, efficient biogas conversion and unhindered system operation. OLR has a critical role in the quantity and composition of the produced biogas (Nkuna et al., 2022). Lower OLRs steer the process to optimum substrate biodegradation and efficient biomethanation, while high OLRs increase the concentration of VFAs, leading to the disruption of the biomethanation process and, in extreme cases, to process failure (Rusanowska et al., 2025).

Co-digestion of agricultural and agro-industrial residues with livestock slurry has emerged as an effective strategy to efficiently degrade organic matter and improve operational parameters (Ferdeș et al., 2023; Karki et al., 2022). Specifically, the co-digestion of recalcitrant lignocellulosic residues, such as rice straw with livestock slurry, is comparatively more advantageous than the mono-digestion of each feedstock separately (Mirabi et al., 2024). Additionally, co-digestion ensures a balanced environment, rich in nutrients, improved C/N ratio, while mitigating the effects of inhibitory compounds (Tian et al., 2023).

Recent studies have explored various pretreatment methods to improve the biodegradability of agricultural residues and increase their methane potential output (Karthikeyan et al., 2024). These pretreatments, including physical, chemical, biological, and combined applications, aim to increase the porosity and surface area of the substrates, disrupt the complex structure of the lignocellulosic biomasses, and thus, make them more accessible to the microbial community (Jankovičová et al., 2025). Sodium hydroxide (NaOH) is one of the most commonly used alkalis for pretreating plant-based feedstocks due to its ability to effectively disrupt the plant's complex structure, increase the porosity of these recalcitrant feedstocks, while being relatively inexpensive and easy to retrieve (Sharma et al., 2019).

Moreover, ammonia, at optimal concentrations, is a critical source of nitrogen for the growth of the anaerobic microbes (Rajagopal, 2025). However, excessive ammonia concentration can inhibit the microbial activity of the methanogenic archaea. Total ammonia concentrations between 1000 and 3000 mg L^{-1} can seriously disrupt the methanogenesis process, depending on pH, while concentrations exceeding 3,000 mg L^{-1}, regardless of pH, can critically inhibit methanogenesis and in some cases cause the entire process to fail (Gaspari et al., 2024).

Moreover, the pH fluctuations are most commonly linked with the concentration of VFAs in the digester (Cioabla et al., 2012). High accumulation of VFAs leads to a lower pH and serves as a sign of process disruption. However, during the commencement of the AD process, known as the startup period, lower pH values and higher VFA accumulation can be observed as the microbial community acclimates to the new feedstock and operational conditions (Santinello et al., 2024).

Despite the growing interest in the co-digestion of lignocellulosic residues with livestock slurry and the established importance of OLR management, there remains limited mechanistic and operational evidence on how the mode of OLR escalation (gradual acclimation versus stepwise loading) affects methane productivity, stability, and recovery capacity in continuous anaerobic systems treating pretreated crop residues. In particular, studies addressing rice straw–swine slurry co-digestion rarely compare gradual and stepwise OLR increases under identical operating conditions, while the combined role of intermediate metabolite dynamics and ammonia-related inhibition during overload remains insufficiently quantified. To address this gap, the present study evaluated the performance of three mesophilic CSTRs co-digesting NaOH-pretreated rice straw with swine slurry, using a control reactor at constant OLR and two experimental reactors subjected to either gradual or stepwise OLR increases. The objective was to determine how different OLR increase strategies influence methane yield, biogas composition, and process stability indicators (VFA accumulation, pH buffering, TAN/FA levels, and COD removal), with emphasis on system resilience and recoverability following overload stress. This work provides practical operational insight for optimizing loading strategies in manure–lignocellulose co-digestion and contributes to safer scale-up by identifying conditions that promote microbial adaptation and minimize instability during high-rate digestion.

2. Materials and methods

2.1 Substrates and inoculum

Rice straw was collected from a paddy at the Institute of Plant Breeding and Genetic Resources (IPGRB) experimental station in Kalochori, Greece. After harvesting, rice straw was collected and stored in a freezer (−20 °C). Before experimentation, rice straw was ground to 0.5 mm particles using a laboratory mill (Retsch, model ZM 1000, Germany) and pretreated with NaOH, as described in our previous study (Kalaitzidis et al., 2026). In summary, rice straw was ground with a laboratory mill and incubated for 24 h with 6% w/w TS NaOH.

Swine slurry was collected from Lagkada SA biogas plant, located in Lagkadas, Greece. The slurry was sieved to remove any large aggregates, using a 1 mm sieve. After sieving, the slurry was placed in 10 L plastic containers and retained in the freezer (−20 °C) for future use. The physicochemical characteristics of rice straw and the swine slurry are presented in Table 1. The inoculum derived from a mesophilic lab-scale batch experiment entailing the co-digestion of pretreated rice straw and swine slurry (Kalaitzidis et al., 2026). The inoculum was retained in an incubator for 10 days under mesophilic conditions (37 ± 1 °C). The TS and VS content of the inoculum was 1.81 and 1.08%, respectively, while the pH was recorded at 7.8.

Table 1. Physicochemical characteristics of the rice straw and swine slurry used in the current study

Feedstock characteristics	Rice straw	Swine slurry
TS (%)	93.25	7.53
VS (%)	81.07	4.35
Ash (%)	12.18	3.19
TOC (%)	38.7	35.48

TN (%)	0.79	2.62
Moisture (%)	6.75	92.47
pH	8.2	7.9

Note: TS = Total solids, VS = Volatile solids, TOC = Total organic carbon, TN = Total nitrogen

2.2 Experimental design

In order to evaluate the effects of gradual and stepwise increases in organic loading rate (OLR) on methane production, three continuous stirred tank reactors (CSTR) with a total volume of 2 L and working volume of 1.5 L were installed at the Waste Management and Bioprocessing (WMB) laboratory of the Hellenic Agricultural Organization – DIMITRA, in Thessaloniki, Greece. (Figures 1,2).

Figure 1. The three continuous stirred tank reactors (CSTR) used in the current experiment. The reactor on the left is R1, the middle one is R2, and on the right is R3.

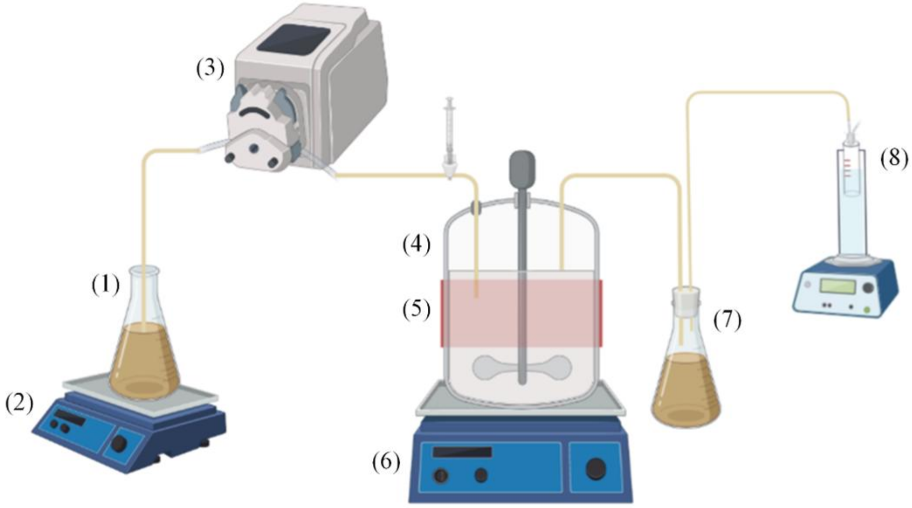

Figure 2. Experimental setup of a continuous flow reactor: (1) Feeding bottle; (2) Feeding bottle magnetic stirrer; (3) Peristaltic pump; (4) Reactor; (5) Heating mantle; (6) Reactor magnetic stirrer; (7) Effluent collection bottle; (8) Biogas meter.

Based on the results of our previous study (Kalaitzidis et al., 2026), the feed mixture of the reactors consisted of 70% swine slurry (%VS) and 30% pretreated rice straw (%VS). The experimental procedure was divided into three periods, each comprising 3 HRTs of 25 days each.

The first reactor (R1) functioned as the control and was maintained at a constant OLR throughout the experiment. The second reactor (R2) was treated with gradual increases, while the third reactor (R3) was subjected to stepwise increases. Throughout the experiment, samples were taken from the reactors to measure all relevant biochemical parameters. Particularly, biochemical parameters such as the concentration of volatile fatty acids (VFA) and pH were measured 3 times per week, while the composition of the biogas produced from each reactor was also determined at the same frequency. Biogas production was recorded on a daily basis. TAN levels were measured throughout the three periods of the experiment, mainly before and after the commencement of OLR increases. Equation 1 was used to calculate the free ammonia concentration (Gaspari et al., 2024).

$$(FAN) = \frac{tan}{1+\frac{10^{-pH}}{Ka}} \qquad \text{Equation (1)}$$

where TAN = total ammonia nitrogen, Ka = dissociation constant with the value of 1.29×10^{-9} at mesophilic conditions.

2.3 Reactor operation

During the first period, all three reactors operated under an OLR equal to 2 gVS L^{-1} d^{-1}. In the second period, the first reactor continued to operate under 2 gVS L^{-1} d^{-1}, while the OLR in the R2 and R3 increased to 3 gVS L^{-1} d^{-1} and 4 gVS L^{-1} d^{-1}, respectively. During the third period, R1 continued to operate under 2 gVS L^{-1} d^{-1}, while the OLR in R2 and R3 increased to 4 and 6 gVS L^{-1} d^{-1}, respectively. The characteristics of the four organic loads are presented in Table 2.

Table 2. Characteristics of the four different OLRs used in the current study

OLR ($gVS\ L^{-1}\ d^{-1}$)	TS (%)	VS (%)	COD ($mg\ L^{-1}$)	C/N
2	5.9	79.4	49.50	
3	8.7	78.1	96.70	22
4	12.0	78.3	129.00	
6	17.6	69.7	161.25	

Note: OLR = Organic loading rate, TS = Total solids, VS = Volatile solids, COD = Chemical oxygen demand

2.4 Analytical methods

The parameters of Total Ammonia Nitrogen (TAN), TS, VS, Ash, Chemical Oxygen Demand (COD), and moisture content were estimated according to standard methods (APHA, 2017). pH was determined with a pH meter (HI2020–02, HANNA instruments, USA). Methane content was determined by utilizing gas chromatography with a Porapak Q column and helium as the carrier gas (GC-TCD detector, Shimadzu, Japan), as described by Mitraka et al. (2022). VFA measurements were performed in a gas chromatograph (GC-2010, Shimadzu, Japan) equipped with a Flame ionization detector (FID). The compounds were separated and quantified by a capillary column (ZBFFAP, 30m, 0.53 mm I.D. × 1 μm film thickness) as described by Xirostylidou et al. (2024). Analyses for C and N were conducted using a Vario EL element analyzer (Elementar Analysensysteme GmbH, Germany).

2.5 Statistical analysis

Analyses of variance (ANOVA) were performed using the MSTAT-C ver. 1.41 (Michigan State University, East Lansing, MI, USA) statistical package. Fisher's least significant difference (LSD) test was employed to detect statistically significant differences between the methane yields of the different reactors in each period, at $\alpha = 0.05$. In addition, Pearson's correlation coefficient (r) was calculated to evaluate the linear relationship between total ammonium nitrogen and methane yield. Correlation analysis was conducted using SPSS ver. 21.0 statistical package (IBM Corp., Armonk, NY, USA).

3. Results and Discussion

3.1 Effect of OLR on the production of intermediate metabolites

In our study, the highest concentration of VFAs (especially propionic and acetic acid) was observed during the first period of the experiment (Figure 3). Specifically, R1, R2, and R3 exhibited the highest concentration of propionic acid, 14 days (R1) and 16 days (R2 and R3) after the beginning of the experiment, and was equal to 2,854, 2,828, and 3,104 mg L^{-1}, respectively. Moreover, the highest concentration of acetic acid in R1, R2, and R3 was equal to 2,909, 2,098, and 2,592 mg L^{-1}, respectively, and was observed on day 5 (R1) and 7 (R2 and R3) after the start of the experiment (Figure 3).

During the second period, the concentration of VFAs in R1 and R2 remained stable and low (~60 mg L^{-1}). In contrast, their concentration in R3 increased considerably. Particularly, the highest increase in acetic and propionic acids' concentration (1,737 and 788 mg L^{-1}, respectively) was exhibited at day 90, while the recovery time was estimated at ~30 days. During the third period, and despite the increases in the OLR of R2 and R3, the VFA of the three reactors remained relatively stable (~60, 150, and 233 mg L^{-1}, respectively) (Figure 3).

VFAs are intermediate metabolites, and their concentration can illustrate the efficiency of the biodegradation process. High VFA accumulation can indicate that the AD process could be under strain (Maurus et al., 2023). At the initial stage of our experiment (~30 days), the accumulation of VFAs, especially propionic and acetic acid, was particularly high (Figure 3). However, in every AD experiment, there is a startup period during which the microorganisms acclimate to the new operational conditions, leading to an increase in VFA accumulation (Santinello et al., 2024).

The high propionic acid concentration was presumably due to the presence of rice straw. Propionic acid and acetic acid form when cellulose and hemicellulose hydrolyze to simple sugars (such as glucose, xylose, mannose, and galactose) (Sobuś et al., 2024). Acetic acid is also the main product of the acetogenesis process, further elevating its concentration in the digester (Rezapoor and Rahimpour, 2024).

The increases in propionic and acetic acids during the second period in R3 can be directly linked to the stepwise increases in OLR. In particular, according to several studies, sudden increases in the OLR can lead to significant VFA accumulation (Magdalena et al., 2019; Rusanowska et al., 2025). However, the gradual increase in the OLR of R2 allowed the system to seamlessly adapt without significant increases in VFA concentration, in accordance with other researchers who have reported similar results (Magdalena et al., 2019).

The robustness and recoverability of our system can be, at least partially, attributed to the high functional redundancy of the acidogenic bacteria (Li et al., 2016) and because mesophilic digesters are more adaptive and perform better at elevated OLRs compared to thermophilic digesters (Gao et al., 2018).

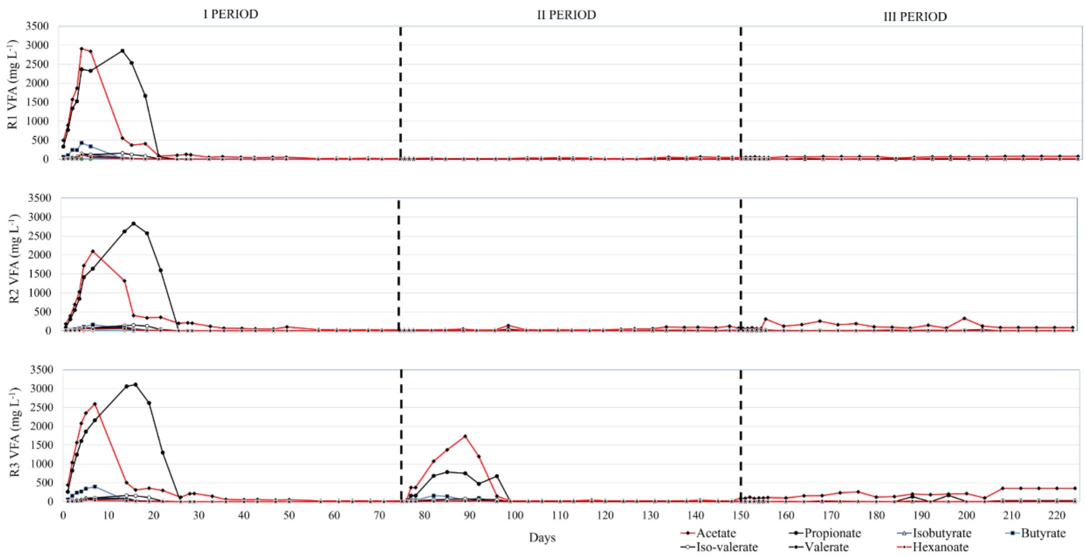

Figure 3. VFA accumulation in the three reactors (R1, R2, and R3) during the three experimental periods. During the first period, all the reactors received 2 gVS $L^{-1}d^{-1}$; in the second period, R2 and R3 received 3 and 4 gVS $L^{-1}d^{-1}$, while in the third period, R2 and R3

received 4 and 6 gVS $L^{-1}d^{-1}$, respectively. R1 was used as the control and continued to operate under 2 gVS $L^{-1}d^{-1}$ throughout the experiment.

3.2 Effect of OLR on methane yield and biogas composition

Methane production in the three reactors (R1, R2, and R3) during the first period followed a similar trend (Figure 4). Specifically, during the initial phase of the experiment (approximately 30 days), methane production increased, and after the ~30th day, and until the end of the first period, methane production stabilized, yielding 347, 348, and 350 ml CH_4 gVS^{-1} from R1, R2, and R3, respectively (Figure 4). According to the statistical analysis, methane yield did not exhibit significant differences among the three reactors (Table 3). A similar trend was observed regarding the percentage of methane in the biogas of the three reactors. Particularly, the percentage of methane in R1, R2, and R3 was 64.7, 64.6, and 64.4%, respectively (Figure 4).

During the second period, methane production remained stable in R1 and R2, while R3 exhibited a significant reduction for approximately 30 days (Figure 4). Nonetheless, after the ~100th day, methane production in R3 stabilized and appeared comparable to R1 and R2 (Figure 4). In particular, methane yield in the three reactors was 353, 351, and 349 ml CH_4 gVS^{-1}, respectively, while no statistically significant differences appeared among them (Table 3). The percentage of methane in the biogas of R1 and R2 was 65.6 and 63.8%, while for R3 it was equal to 63.1% (Figure 5).

In the third period, reactors R1 and R2 appeared to have similar methane yields, while methane production in R3 was significantly impaired (Figure 4). Specifically, R1 and R2 produced 350 and 348 ml CH_4 gVS^{-1}, respectively, while R3 yielded 331 ml CH_4 gVS^{-1} (Figure 4). Simultaneously, the methane content in R1 and R2 remained relatively stable at 64.5 and 63.2%, respectively, while the percentage of methane in R3 decreased to 51%. Moreover, the decrease in methane production of the third reactor was statistically significant (Table 3). From Figure 5, we can deduce that the stepwise increase in the OLR resulted in a substantial decrease in CH_4 production and an equal increase in CO_2 production.

Several studies have reported similar results, indicating the superiority of gradual increases in OLR compared to stepwise increases. Gradual increases in OLR allow the microbial community to slowly adjust to the new organic load, while sudden changes lead to severe process disruption and longer adaptation periods (Ahmad et al., 2021; Ketheesan and Stuckey, 2015). Moreover, other researchers have recorded similar values of methane production when rice straw is pretreated and co-digested with swine manure (Zhong et al., 2021).

The reduction in methane production in R3 during the second period was likely due to the stepwise increase in OLR, resulting in a high accumulation of VFAs and a subsequent decrease in methane production (Rusanowska et al., 2025; Wu et al., 2022). The high accumulation of VFAs leads to lower pH, disruption of the microbial equilibrium, and can be toxic for the methanogenic and acidogenic microorganisms (Shyan et al., 2023).

In the third period, methane production in R1 and R2 remained stable, showing the excellent adaptation of R2 to the new organic load. R3 demonstrated stable methane production with a simultaneous increase in CO_2 production. This shift in the CH_4/CO_2 ratio can be the result of stress, especially from ammonia toxicity (Bucci et al., 2024; Zhuang et al., 2018).

Table 3. Statistical comparison of the methane yield produced by the three reactors during the three periods of the experiment.

Reactors	Methane yield (ml CH_4 gVS^{-1})		
	Period I	Period II	Period III
R1	347^{a}	353^{a}	350^{a}
R2	348^{a}	351^{a}	348^{a}
R3	350^{a}	349^{ab}	331^{b}

Different letters indicate statistically significant differences at $p < 0.05$.

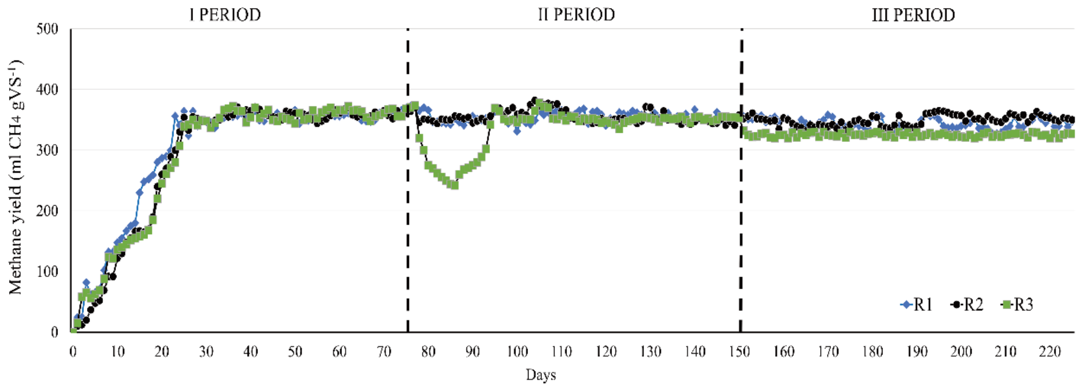

Figure 4. Methane yield (ml CH_4 gVS^{-1}) of the three reactors during the three periods of the experiment.

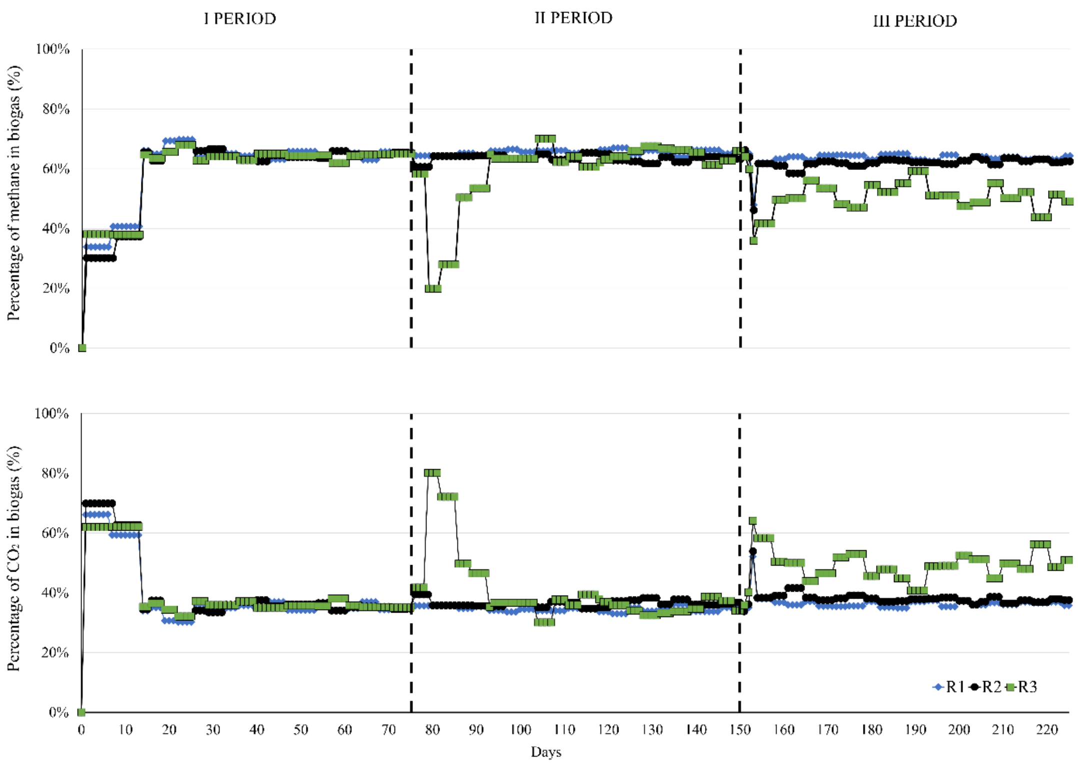

Figure 5. Percentage of methane (CH_4) and carbon dioxide (CO_2) in the biogas of each reactor (R1, R2, and R3), during the three experimental periods.

3.3 Analysis of the stability of the AD process

3.3.1 Effect of OLR on pH, VFA, and system buffer capacity

pH patterns closely mirrored VFA accumulation throughout the experimental process. Initially, all reactors showed a decline in pH and an increase in VFA. Maximum increases in VFA were observed on days 7 (R1, R3) and 14 (R2) and were equal to ~6,000, ~5,500, and ~4,400 mg L^{-1}, respectively (Figure 6). Likewise, the lowest pH values were recorded at the same interval (~7.4). After approximately 40 days, pH values and VFA concentrations stabilized at ~7.6 and ~60 mg L^{-1}, respectively.

During the second period, pH levels in R1, R2, and R3 were further elevated to ~8.0, while VFA accumulation in R1 and R2 remained stable. In contrast, pH in R3 started to decrease, while VFA accumulation started to increase. After ~15 days (day ~90), pH in R3 reached its lowest recorded value equal to 7.5, while VFA recorded its highest accumulation (2,728 mg L^{-1}). The recovery phase lasted ~30 days, and in the continuum, the TVFA and pH stabilized and appeared comparable with the other two reactors.

During period III, all reactors exhibited similar pH (~8.0). VFA concentration remained stable in R1, while R2 and R3 experienced marginal increases. In particular, VFA in R2 and R3 increased from ~60 to ~150 and ~233 mg L^{-1}, respectively.

During the first period, the operation successfully adapted to the new operational conditions, retaining pH values within the limits for anaerobic digestion (Filer et al., 2019). The increase in VFA and subsequent pH decline during the initial stages of the experiment can be linked with the increased rate of acid production and the lower rate of acid consumption during the start-up period (Nikitina et al., 2023). The relatively high pH (> 7.2), despite significant VFA accumulation, was likely due to the buffering capacity of our co-digestion system. During the third period, slight increases in the VFAs of R2 and R3 were observed. However, while these increases were limited, they could imply potential stress from ammonia accumulation, as suggested by Gaspari et al. (2024).

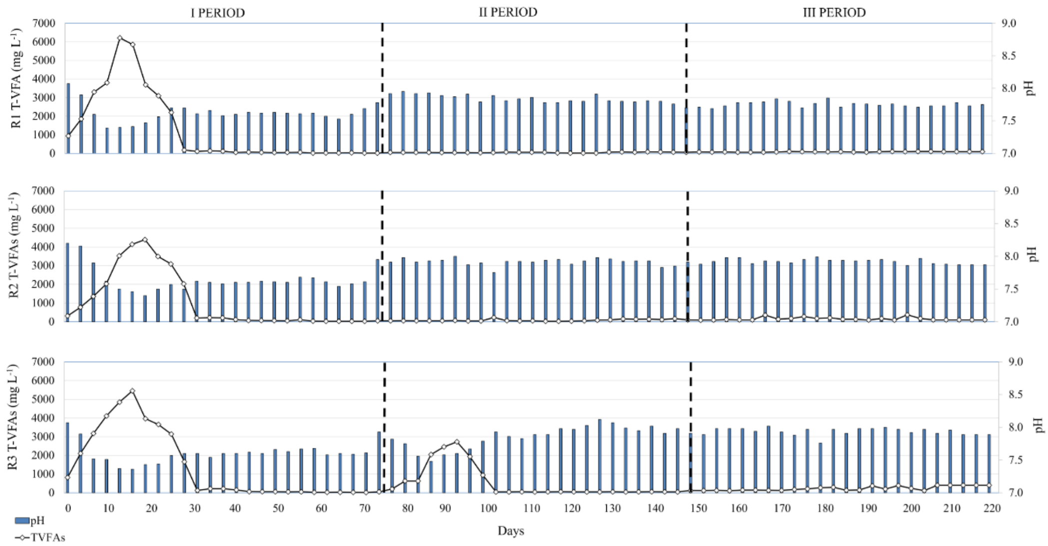

Figure 6. Evolution of pH and TVFA in reactors R1, R2, and R3 during the three periods of the experiment. Bars indicate pH values, and lines with markers show VFA accumulation.

3.3.2 Ammonia inhibition and microbial adaptation

Total ammonia nitrogen during the initial stages of the first period remained relatively high and close to 2,000 mg L^{-1} in all reactors. However, after ~40 days, the concentration of ammonia steadily declined below 2,000 mg L^{-1}, with R2 even recording values lower than 1,500 mg L^{-1} (Figure 7). At the commencement of the second period (days 75 to ~100), TAN values spiked over 2,500 mg L^{-1} in R2 and close to 3,000 mg L^{-1} in R3, following the increases in OLR, while recorded values in R1 remained stable and close to 1,660 mg L^{-1}. After day 100, the concentration of total ammonia began to drop and stabilized at approximately 2,000 mg L^{-1} for both R2 and R3 (Figure 7).

In period III, recorded values of TAN in R3 began to elevate, stabilizing at just under 3,500 mg L^{-1}, approximately 50 days after the start of the third period (day ~200). Values of TAN in R2 presented a slight increase for approximately ~30 days and then commenced to decline, stabilizing at approximately 2,200 mg L^{-1}, while TAN values in R1 remained stable (~1,600 mg L^{-1}) (Figure 7).

According to the literature, total ammonia concentrations between 1,000 and 3,000 mg L^{-1} can induce inhibitions in the AD systems, depending on pH levels, while values exceeding 3,000 mg L^{-1} have been known to be toxic regardless of pH levels (Gaspari et al., 2024). However, recent studies have shown that co-digestion of animal manure with agricultural and agro-industrial wastes can enhance the tolerance of the system to elevated ammonia levels.

Specifically, Li et al. (2018) found that the co-digestion of chicken manure with apple pulp presented the highest specific methane yield, attributed to the elevated buffer capacity and the balanced nutrient content of the feedstock. Likewise, Adghim et al. (2021) reported enhanced methane potential from the co-digestion of poultry manure with cheese factory and coffee ground wastes due to reduced ammonia levels.

While TAN encapsulates both forms of ammonia (NH_4^+ and NH_3), FA is considered particularly toxic to the methanogenic archaea (Bucci et al., 2024). The threshold for immediate concern in FA concentrations is 200 mg L^{-1}, while values exceeding 300 mg L^{-1} can induce severe methanogenic inhibition. During period III, the three reactors showed 63, 131, and 212 mg L^{-1} of FA, respectively, with the FA concentration in R3 exceeding the threshold of 200 mg L^{-1} (Rocamora et al., 2025) but remaining below 300 mg L^{-1} (Yenigün and Demirel, 2013). However, exceeding the threshold of 200 mg L^{-1} is enough to cause considerable disruption in the AD process, which could explain the reduction in methane production during the third period compared to periods I and II.

Nevertheless, successful operation of the third reactor at 212 mg L^{-1} of FA and TAN concentrations nearing 3,500 mg L^{-1} without complete failure indicates the system's robustness, consistent with recent findings on enhanced TAN tolerance in anaerobic digesters. In particular, Beraud-Martínez et al. (2024) demonstrated that gradual ammonia exposure leads to successful recovery of the methanogenesis process, even under high TAN concentrations.

Moderate stress in the AD process, induced by ammonia toxicity, can shift methanogenesis from predominantly acetoclastic to hydrogenotrophic (Bucci et al., 2024). This transition is characterized by a decrease in the methanogenesis efficiency and an increase in CO_2 release (Zhuang et al., 2018). As a result, the ratio of CH_4 and CO_2 in the biogas can be altered in favor of carbon dioxide. As shown in Figure 5, the percentage of CO_2 in R3, during the third period, increased considerably compared to the percentage of CH_4, potentially linking the methane yield reduction in R3 with increases in TAN.

Moreover, the correlation analysis revealed a strong and negative linear relationship between TAN and methane yield ($r = -0.826$, $p < 0.01$), with TAN concentration accounting for 68.2% of the variation in methane yield ($R^2 = 0.682$, adjusted $R^2 = 0.636$). Nakakubo et al. (2008) reported a higher negative correlation ($R^2 = 0.91$), while also noting that ammonia accumulation doesn't necessarily lead to elevated levels of VFAs. Likewise, in our study, elevated levels of TAN did not always result in elevated levels of VFA (Figures 3, 6).

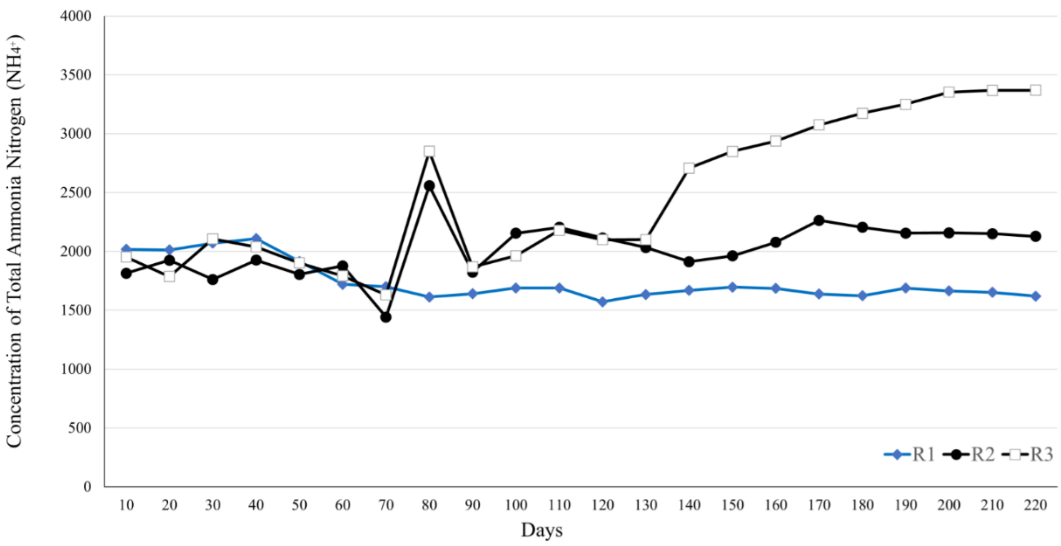

Figure 7. Total ammonia nitrogen (TAN) in reactors R1, R2, and R3 during the three periods of experimentation.

3.3.3 COD Removal Efficiency and Substrate Utilization

COD removal efficiency during the first period was comparable for R1, R2, and R3, equal to 65.7, 65.9, and 65.8%, respectively. During the second period, increases were marginal for R1 and R2 (67.1 and 66.3%), while remaining relatively stable in R3 (65.3%). In period III, R1 and R2 continued to experience limited incremental changes in their COD removal efficiency (67.9 and 66.6%, respectively), while R3 showed a considerable decrease (62.4%). Analogous results were obtained from other researchers performing similar experiments.

Specifically, Darwin et al. (2014) performed a batch-scale experiment comparing co-digestion with mono-digestion of digested swine manure with rice straw in mesophilic conditions. The results showed that the COD removal at the optimum TS concentration was 53%. In another rice straw-pig manure co-digestion experiment, performed by Zhong et al. (2021), the COD removal rate reached 70%. Ahmed et al. (2024) reported a maximum removal efficiency of 70% in an experiment entailing the co-digestion of rice straw and thickened sewage sludge. These results show that the COD efficiency in our experiments was effective, even under stress conditions, attributed to stepwise increases in OLR and elevated TAN levels.

High OLRs also lead to further decreases in COD removal, due to the microorganism's inability to efficiently metabolize excessive loads of organic matter (Alotaibi et al., 2024; Aris et al., 2017). Hence, R1 consistently demonstrated the highest COD removal efficiency across all periods. Conversely, R2 exhibited only marginal increases, while R3's efficiency remained stable in Period II and decreased considerably in Period III.

4. Conclusion

This study confirms that co-digestion of swine slurry with alkali-pretreated rice straw (70%:30% VS) is an efficient and robust strategy for biogas production in mesophilic CSTR operation. Across all reactors, the highest and most stable performance was achieved at an OLR of 2 gVS L^{-1} d^{-1}, reaching a maximum methane yield of 353 mL CH_4 gVS^{-1} with a methane content of approximately 65%, demonstrating strong conversion efficiency for this lignocellulosic–manure feedstock combination. A central contribution of this work is showing that the mode of OLR increase strongly governs stability and methane productivity. Gradual OLR increases (up to 4 gVS L^{-1} d^{-1}) caused only transient declines, indicating strong acclimation and rapid recovery, whereas stepwise loading, especially at 6 gVS L^{-1} d^{-1}, significantly reduced methane yield (331 mL CH_4 gVS^{-1}) and lowered methane concentration to ~51%. Process impairment under shock loading was closely linked to ammonia stress, with TAN rising to ~3500 mg L^{-1} and free ammonia reaching 212 mg L^{-1} ($r = -0.826$ vs methane yield). Notably, COD removal remained relatively resilient (65–67%), underscoring the robustness of this co-digestion system. Overall, controlled OLR escalation is essential to maximize energy recovery and avoid ammonia-driven inhibition.

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Chapter 5: Investigating the effect of anaerobic digestion residues on basil growth, secondary metabolite synthesis and growing substrate properties

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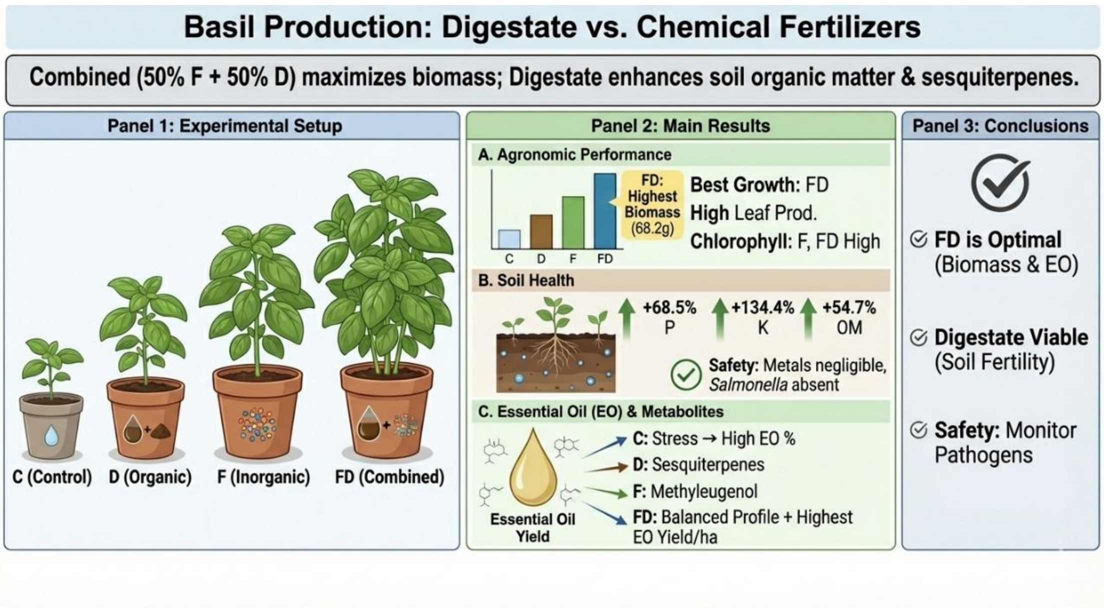

Abstract

To assess digestate's efficacy as a fertilizer for basil development, a two-year pot experiment was established, comprising four fertilization treatments: chemical fertilizer (F), digestate (D), combined chemical fertilizer and digestate (1:1, FD), and unfertilized control (C). Key metrics assessed included: plant height, chlorophyll concentration index (CCI), total biomass (TB), leaf production (LP), essential oil yield, and composition. Post-harvest soil analysis evaluated nutrient and heavy metal content and pathogen contamination in soil and leaves. FD treatment produced the highest TB (68.2 g plant^{-1}) and LP (52.7 g plant^{-1}). Digestate application substantially enhanced substrate nutrient availability, increasing extractable phosphorus by 68.5%, potassium by 134.4%, and O.M. by 54.7%. Essential oil yield was significantly higher in the control plants. Notably, different fertilization regimes altered secondary metabolite synthesis. Specifically, fertilization with digestate favored sesquiterpenes synthesis, inorganic fertilization enhanced methyleugenol and β-farnesene synthesis, while the control showed higher limonene, eugenol, and linalool. Heavy metal accumulation in the soil was negligible, remaining well within regulatory limits. *Salmonella spp.* were not detected. *Enterococcus faecalis* and *Escherichia coli* were present in the soil in low concentrations and marginally below EU safety limits (100 cfu g^{-1}) on the leaves.

1 Introduction

Agricultural sustainability in the Mediterranean region is facing significant issues due to soil degradation and desertification, attributed to climate change and land mismanagement (Ferreira et al., 2024). Simultaneously, a considerable reduction in precipitation and an increase in salinization are expected to occur, undermining soil fertility in the Mediterranean region (Ferreira et al., 2024; Seker and Gumus, 2022). To address these challenges, digestate, the by-product of anaerobic digestion (AD), has emerged as a promising organic fertilizer that can restore and enhance soil quality.

Fertilization with digestate has the potential to increase soil fertility by stimulating the propagation of beneficial microorganisms, promoting microbial community diversification, and enhancing carbon sequestration (Roopnarain et al., 2023). Moreover, digestate application improves soil physical structure by increasing the number and mean weight diameter (MWD) of 5–10 mm and 1–5 mm water-stable aggregates, while reducing the smaller ones (<1 mm).

These improvements in soil aggregation have been observed to persist for up to four years post-application, demonstrating digestate's potential for sustainable soil improvement (Pranagal et al., 2023).

Furthermore, long-term digestate application significantly enhances soil health and mechanical properties. Holatko et al. (2023) reported that digestate application increased soil sulfur content, enhanced the activity of key enzymes involved in nutrient cycling (including arylsulfatase, N-acetyl-β-D-glucosaminidase, and phosphatase), and improved nitrogen sequestration over extended periods. Didelot et al. (2023) demonstrated that continuous digestate application resulted in significantly higher dissolved organic carbon (DOC) fluxes than mineral fertilizers or raw pig manure, highlighting the superior soil-enhancing capacity of digestate. Additionally, digestate application influences nutrient retention and recycling, making it a more efficient fertilizer option for diverse cropping systems (Didelot et al., 2023).

However, digestate application raises potential safety concerns. Specifically, digestate produced from animal manure can contain pathogens, such as *Salmonella spp.*, *E. coli*, and *Enterococcus faecalis*, which are sometimes able to withstand the digestion process (Ma et al., 2022; Seruga et al., 2020). Carraturo et al. (2022) reported that thermophilic digestion sanitizes digestate more effectively than mesophilic digestion, due to the elevated temperatures present during the thermophilic AD process. Indicating that all digestates, and particularly those from mesophilic conditions, require monitoring to ensure sufficient elimination of pathogenic microbes.

Heavy metal contamination presents another concern, as animal feed can introduce elevated concentrations of metals such as Cd, Cr, Ni, Pb, Zn, and Cu (Hejna et al., 2018). This risk is further exacerbated by digestate's relatively low nutrient content, necessitating higher application volumes to achieve nutrient levels comparable to mineral fertilizers (Derehajło et al., 2023). Consequently, repeated or excessive digestate applications may lead to heavy metal accumulation in the soil (Ma et al., 2022). The introduction of excessive pollutant loads through low-quality waste applications can adversely affect soil fertility, threaten groundwater quality, and contaminate the food chain (Gao et al., 2024; Yunta et al., 2024).

Consequently, evaluating digestate’s performance on diverse crops is essential to assess both the agronomic benefits and the safety challenges. Digestate has been successfully employed as fertilizer for various medicinal and aromatic plants (MAPs), including rosemary, peppermint, lemongrass, and basil, with positive effects on both biomass production and the synthesis of secondary metabolites (Barra Caracciolo et al., 2015; Ronga et al., 2018; Rowe et al., 2023). In particular, Rowe et al. (2023) reported that the fertilization of lemongrass with digestate increased the overall biomass production eightfold, compared to the unfertilized control. Ronga et al. (2018) found that the combined application of solid digestate as a growing medium and liquid digestate as the nutrient solution increased shoot dry weight, total dry weight, and the relative amount of sesquiterpenes in basil and peppermint plants grown hydroponically. Moreover, digestate application led to significant increases in the number of leaves, total number of branches, plant dry weight, and essential oil content in rose-scented geranium plants (*Pelargonium graveolens* L’Hér.) (Calamai et al., 2019).

Basil (*Ocimum basilicum* L.) is one of the most economically important MAPs, widely used in Mediterranean cuisine and across the globe (Brindisi and Simon, 2023). Basil is consumed fresh or dried as a culinary herb, while its essential oil is used in the food, pharmaceutical, and flavoring industries (Brindisi and Simon, 2023). Essential oil from basil exhibits exceptional medicinal properties, including antiviral, antibacterial, antioxidant, antifungal, and anticancer activities (Azizah et al., 2023), while its main constituents comprise linalool, eucalyptol, estragole, and eugenol (da Silva et al., 2022).

The present study aimed to investigate the effect of digestate on key agronomic characteristics and the growing substrate of basil. Notably, it is the first study to investigate the effect of digestate on the essential oil composition of basil plants cultivated in soil.

2. Materials and methods

2.1 Plant materials and experimental design

The experiments were conducted at the Institute of Plant Breeding and Genetic Resources (IPGRB), in Thessaloniki, Greece (Latitude 40°36′58.75″ N, Longitude 22°49′51.16″ E). The experiment was conducted in a net-house with a transparent polycarbonate roofing (10 mm thickness, 2.10 m × 6.00 m). The roofing was placed to ensure protection from rainfall and controlled irrigation conditions. Notably, plants were grown under natural light throughout the experimental process.

Seeds of a large-leaved sweet basil (*Ocimum basilicum* L.) germplasm (preserved in the IPGRB collection) were sown in 96-cell plastic trays containing peat substrate in mid-March 2023 and 2024 (Figure 1).

(a) (b)

Figure 1. a) Newly emerged basil plants; b) Basil plants with four fully developed leaves ready for transplanting

Approximately 30 days after sowing (DAS), seedlings were individually transplanted into 2.5 L polyethylene pots at the four-leaf stage (Figure 2).

Figure 2. Basil plants transplanted into 2.5 L polyethylene pots.

The growing substrate comprised soil and perlite at a 2:1 ratio (v:v). Soil was collected from the top 0–30 cm layer of a field near the IPGRB campus, used for MAP cultivation. After collection, the soil was sieved with a 2 mm mesh to remove rocks, large aggregates, and other extraneous materials. The soil was classified as sandy loam (SL) with a pH of 8.5, low in heavy metals, and free of pathogens (Table 1).

Table 1. Physicochemical properties, heavy metal, and pathogen content of the soil and digestate used in the current experiment.

Physicochemical Properties	Units	Soil	Digestate
pH	-	8.5	7.8
Electric Conductivity (EC)	$mS\ cm^{-1}$	0.24	2.42
Salinity	psu	0.13	1.55
Organic Matter (O.M.)	%	1.81	-
Soil Texture	-	Sandy Loam (SL)	-
Clay	%	14.1	-
Silt	%	30.8	-
Sand	%	55.1	-
Dry Matter (TS)	%	-	1.81
Volatile Solids	%	-	1.08
Total Nitrogen	%	-	0.2
Phosphorus (P)	$mg\ kg^{-1}$	11.87^{E}	310^{T}
Nitrate Nitrogen ($N\text{-}NO_3$)	$mg\ kg^{-1}$	25.77^{E}	<500
Calcium (Ca)	$mg\ kg^{-1}$	3610^{E}	710^{T}
Magnesium (Mg)	$mg\ kg^{-1}$	290^{E}	260^{T}
Potassium (K)	$mg\ kg^{-1}$	320^{E}	1430^{T}
Zinc (Zn)	$mg\ kg^{-1}$	2.70^{E}	18^{T}
Iron (Fe)	$mg\ kg^{-1}$	5.09^{E}	73.8^{T}
Manganese (Mn)	$mg\ kg^{-1}$	3.53^{E}	11.9^{T}
Boron (B)	$mg\ kg^{-1}$	0.19^{E}	2.31^{T}

Sodium (Na)	mg kg $^{-1}$	113^{E}	560^{T}
Heavy Metals			
Cadmium (Cd)	mg kg $^{-1}$	0.18^{T}	0.007^{T}
Chromium (Cr)	mg kg $^{-1}$	75.5^{T}	0.35^{T}
Mercury (Hg)	mg kg $^{-1}$	0.05^{T}	0.02^{T}
Copper (Cu)	mg kg $^{-1}$	23.7^{T}	0.40^{T}
Lead (Pb)	mg kg $^{-1}$	14.0^{T}	0.09^{T}
Arsenic (As)	mg kg $^{-1}$	7.90^{T}	0.05^{T}
Pathogens			
Salmonella spp.	cfu g^{-1}	N.D.	N.D.
Enterococcus faecalis	cfu g^{-1}	N.D.	1,8 x 10^{2}
Escherichia coli (E. coli)	cfu g^{-1}	N.D.	45est

T = Total, E = Extractable

To assess the efficacy of digestate as an alternative organic fertilizer for developing basil, a randomized complete block design (RCBD) experiment was employed with 3 replicates, comprising 6 plants *per* replication and 4 fertilization regimes, for a total of 72 plants. The fertilization treatments applied were: 1) digestate (D); 2) combined digestate with inorganic fertilizer (FD), where plants received 50% of their nitrogen requirements from digestate and 50% from inorganic fertilizer; 3) liquid fertilizer (F); and 4) unfertilized control (C).

The digestate used in the current study was obtained from an anaerobic digestion batch experiment performed in the Soil and Water Resources Institute (SWRI) in Thessaloniki, Greece (Kalaitzidis et al. 2025). The experiment entailed the use of rice straw and swine slurry at a percentage ratio of 30%:70% (on a volatile solids basis), as feedstock for an anaerobic digester operating in mesophilic conditions (37 ºC). The physicochemical characteristics of the digestate, the heavy metal, and pathogen content are presented in Table 1. Prior to fertilization, the digestate's pH was adjusted to neutral (7), using 1 M HCl.

The inorganic fertilizer used in the current experiment was commercially available and designed for green leafy plants,7-3-6 NPK (Compo, Münster, Germany). It contained 7% total Nitrogen, comprising 2.6% N-NO_3, 1.5% N-NH_4, and 2.9% N-NH_2. Moreover, it included 3% P_2O_5, 6% K_2O, and other micronutrients.

During experimentation, all plants were irrigated twice per week using tap water and maintained at 70% Water Holding Capacity (WHC), estimated gravimetrically. All fertilized plants received equal amounts of nitrogen (2.5 g plant^{-1}), although the source of nitrogen differed among treatments. Fertilizers were applied with each irrigation event (twice weekly) in equal portions throughout the growing period, beginning one week after transplanting. Nitrogen fertilization was calculated as 250 kg N ha^{-1} (Bufalo et al., 2015), with a plant density of 10 plants *per* square meter. Although most studies recommend lower rates, we selected the highest recommended fertilization to make the benefits and risks from digestate application more pronounced.

At 60 DAS, plant inflorescences were removed to promote the formation of lateral branches (Dun et al., 2006). The plants were harvested at 90 DAS by cutting the stems at the soil surface. The leaves were removed and stored at -20 °C for subsequent analyses of essential oil yield, essential oil composition, and concentration of pathogens. Additionally, at the end of experimentation, the growing substrates of all the pots from each treatment were combined and homogenized. Two samples (1 kg each) per replicate were collected and stored at -20 °C for nutrient, heavy metal, and contaminant analysis. The experimentation was conducted over two consecutive growing seasons (2023, 2024).

2.2 Environmental conditions

Temperature and relative humidity were continuously monitored using a HOBO Micro Station (Onset Computer Corporation, Bourne, MA, USA). The climatic conditions for 2023 and 2024 are presented in Figure 3. The differences between min, max, and average temperature, along with relative humidity, were minor.

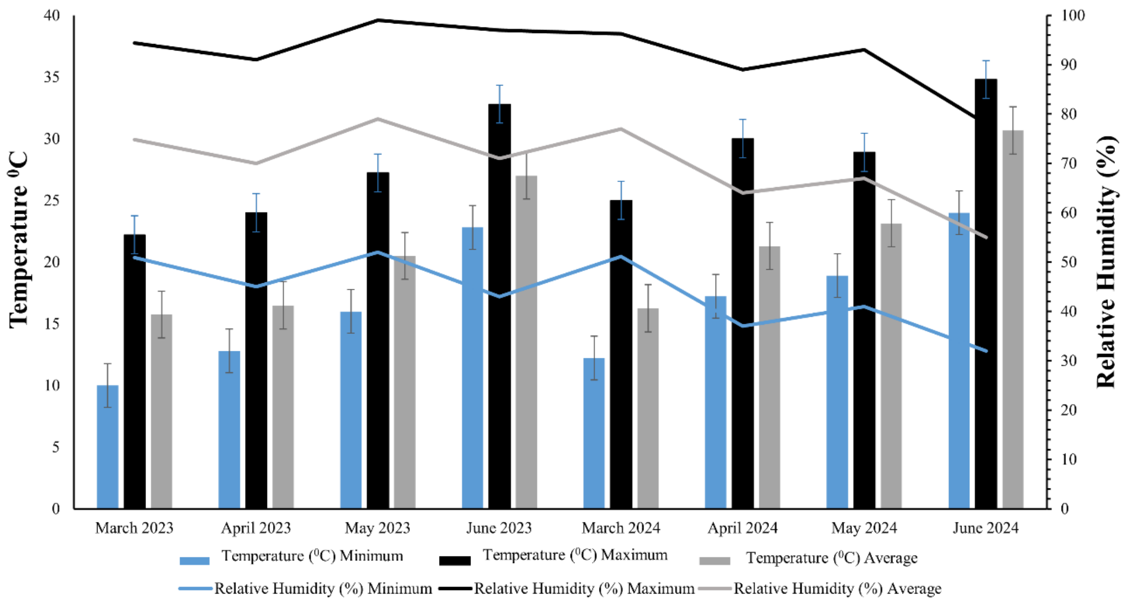

Figure 3. Monthly Temperature and relative humidity during the two growing seasons in 2023 and 2024 at the experimental site. Bars represent minimum (light blue), maximum (black), and average (grey) temperature, while lines represent minimum (light blue), maximum (black), and average (grey) relative humidity.

2.3 Growth and physiological parameters

2.3.1 Growth parameters

Prior to harvest, plant height was recorded weekly at 39, 49, 55, 62, 69, and 76 DAS by measuring the stems from the ground level up to the tip of the plants. At harvest, total fresh aboveground biomass (TB) and leaf production (LP) at 90 DAS were determined gravimetrically (g $plant^{-1}$).

2.3.2 Physiological parameters

Chlorophyll content (CCI) was measured on the two youngest fully developed leaves of all plants using a portable chlorophyll content meter (CCM-200, Opti-Sciences, Tyngsboro, MA, USA) at 39, 49, 55, 62, 69, and 76 DAS.

2.4 Analytical methods

2.4.1 Analytical methods for physicochemical and microbiological characteristics

The determination of volatile solids (VS) and total solids (TS) was performed according to standard methods (APHA, 2005). To determine the heavy metal concentration, the samples were decomposed in acid at a high digestion vessel pressure with the aid of a Milestone Ethos Up microwave oven (Milestone Srl, Sorisole, Italy), and the resulting solution was analyzed, using an Agilent 7850 ICP-MS (Agilent Technologies, Santa Clara, CA, USA) equipped with the ORS4 collision cell (Baird et al., 2017). Electrical conductivity was evaluated on approximately 10 g of sample, which was placed into a 250 mL plastic container with 50 mL of deionized water. The sample was stirred for 1 h, followed by filtration. In the filtrate, the electrical conductivity was measured at 25 °C. Total nitrogen was calculated according to the standard Kjeldahl method. Nitric nitrogen ($N-NO_3$) was determined by spectrophotometry (HACH DR 3900, Hach-Lange Ltd, Denmark). Total calcium carbonate was determined by gravimetric analysis. The method was based on the reaction of HCl with calcium carbonate and the gravimetric loss of CO_2 from the sample (McGeorge, 1954).

The detection of *Salmonella* was conducted according to Midorikawa et al. (2014). The number of enterococci was determined by plating known amounts of the sample on the surface of a selective medium (*Enterococcus* agar) prepared according to the method of Slanetz and Bartley (1957). After 48 hours of incubation at 44 °C, colonies exhibiting typical morphology were enumerated. The method of detection and enumeration of *Escherichia coli* in the digested material was based on the ISO 16193:2013 (2013) standard.

2.4.2 GC-MS/FID analysis

Leaf samples from the aerial parts were collected from all the plants *per* treatment and combined to constitute three bulk samples (200–300 g each). A Clevenger-type apparatus was used to determine the essential oil (EO) content (mL 100 g^{-1} on a fresh weight basis). The fresh leaves (100 g of pooled sample *per* treatment) of basil were subjected to hydrodistillation for 3 hours, with a distillation rate of 3–3.5 mL min^{-1}. The EO was collected in 4 mL glass vials, dried over anhydrous sodium sulfate, and stored at 4–6 ºC until further analysis. The fresh biomass was distilled in triplicate, and the % yield was expressed as the mean of three independent replicates.

Essential oil samples were diluted in pentane before they were injected for analysis. A Shimadzu 17 A Ver. 3 gas chromatograph interfaced with a QP5050A mass spectrometer and holding a capillary Agilent HP-5MS 30 m, 0.25 mm, 0.25 μm column, and supported by the GC/MS Solution ver. 1.21 software was employed for the analysis of the EO. The conditions of analysis followed were previously described by Sarrou et al. (2017). The abundance of each compound was calculated as the percentage of the total chromatographic area. The identification of the compounds was performed through the comparison of a) their retention indices (RI) with those of n-alkanes (C7–C22) (Adams, 2001), b) with the corresponding literature data, and c) by matching their spectra with those of the MS libraries (NIST 98, Willey). For EO constituents' quantification, GC/FID analysis was carried out on a Shimadzu Nexis GC-2030 series with a flame ionization detector (FID) and a Shimadzu AOC-20i auto injector using a Crossbond MEGA5MS column (30 m X 0.25 mm, film thickness 0.25 μm) coated with 95% methyl polysiloxane. The oven temperature was as follows: 55 °C (hold time 1 min), 55–110 °C (rate 1.5 °C min^{-1}), 110–150 °C (3 °C min^{-1}), 150–220 °C (8 °C min^{-1}), and 220 °C for 10 min. The injector temperature was set at 260 °C and the detector temperature at 280 °C. Injection volume was 1 μL, He was used as a carrier gas (1 mL min^{-1}), and the split ratio was 1:30.

2.5 Statistical analysis

ANOVA was conducted for data analysis using MSTAT-C ver. 1.41 (Michigan State University, East Lansing, MI, USA). Experimentation time (runs) and the interaction between the Time by experimental treatment (T) were not significant; hence, data from the two runs were pooled and subjected to ANOVA analysis. Fisher's least significant difference (LSD) was used to determine significant differences among the examined treatments, at $p < 0.05$ ($n = 6$).

3. Results and Discussion

3.1 Physiological parameters

During the first week of measurements (39 DAS), all treatments exhibited similar heights (12.5–12.8 cm). In the second week, plants receiving inorganic fertilization either exclusively or partially (F and FD) were significantly taller than D-treated and control plants. From the third week onwards, all plants receiving fertilization did not show significant differences among them, while the control consistently presented the shortest plants (Figure 4).

Several researchers have reported that digestate supports plant growth equally well compared to conventional inorganic fertilizers. According to Ronga et al. (2018), the use of liquid digestate as a nutrient solution and solid digestate as a growing medium in hydroponically grown basil plants increased the height of the basil plants considerably, compared to mineral fertilization. Asp et al. (2022) reported that 50% substitution of growing media with digestate, in basil cultivation, performed analogously to conventional fertilized treatments, consistent with our findings.

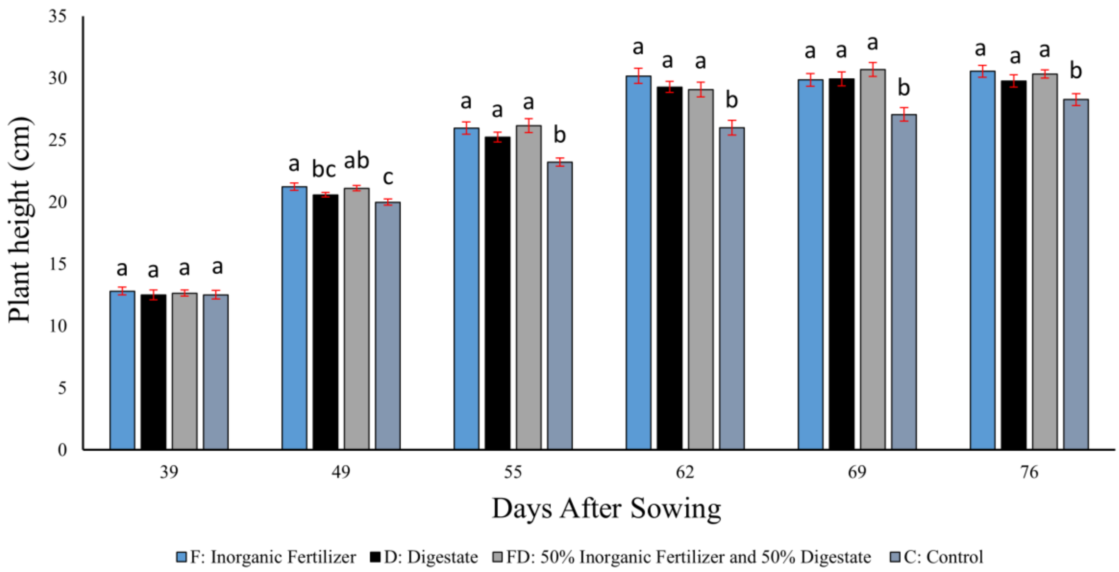

Figure 4. Plant height (cm) of basil plants measured at 39, 49, 55, 62, 69, and 76 days after sowing (DAS), under different fertilization treatments. Values are means over two years. Error bars represent the standard error of means. Within each sampling occasion, different letters above the bars indicate statistically significant differences between treatments at $p < 0.05$.

At 39 DAS, F-treated plants exhibited the highest CCI values (18), compared to all other treatments (13–14). At 49 and 55 DAS, F-amended plants continued to present the highest CCI values (25), while the FD plants ranked second. D-treated plants exhibited intermediate values, and control plants demonstrated the lowest CCI values (13). From 62 DAS onwards, the F and

FD plants did not show significant differences, D-treated plants followed, and control plants consistently maintained the lowest CCI values (Figure 5).

The variation in CCI values according to treatment reflects the differences in nutrient availability depending on the fertilization regime. Specifically, the inorganic fertilizers provide readily available nitrogen that plants utilize for rapid chlorophyll production (Wu et al., 2024), while organic fertilizers release nitrogen slowly as they rely on microbial decomposition (Phillips et al., 2022). Hence, at the beginning of our experiment, plants receiving solely inorganic fertilizer exhibited higher CCI values, while plants treated exclusively with digestate exhibited lower CCI values.

In contrast, basil plants receiving the inorganic-digestate mixture (FD) performed considerably better than D-treated plants and, from 62 DAS onwards, exhibited values comparable to the F-treated plants. Therefore, inorganic and organic fertilizers appeared to work in a complementary manner. The readily available nitrogen from the inorganic fertilizers facilitated immediate chlorophyll synthesis, while digestate provided a slower but continuous stream of nitrogen.

Similar results were reported by Li et al. (2023), who studied the effect of an inorganic fertilizer, digestate, and their combination on tomato plants. According to their results, digestate addition, whether partial or exclusive, led to significant increases in the leaf chlorophyll content of the tomato plants.

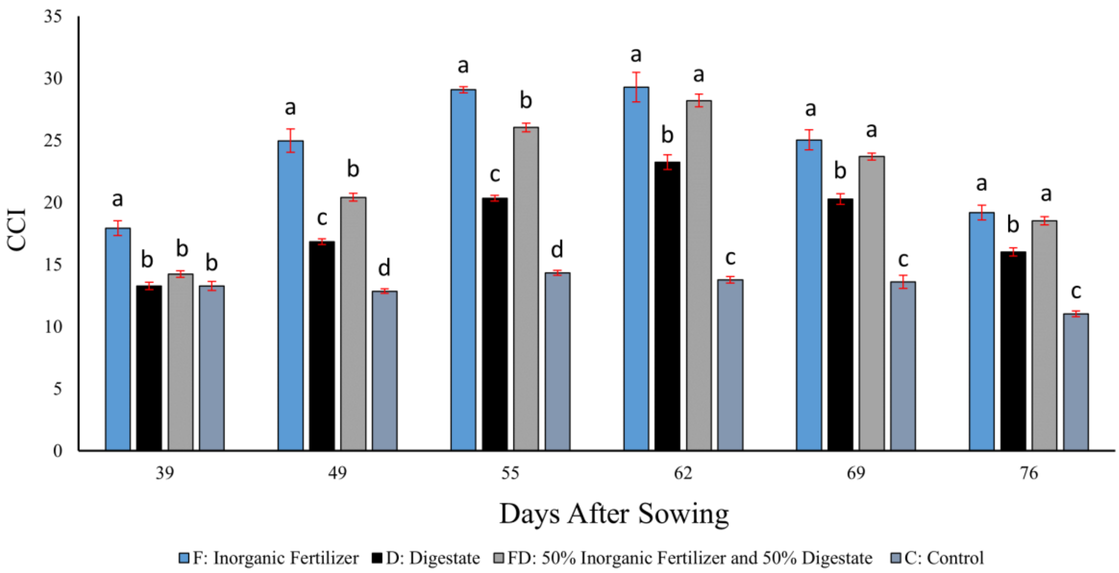

Figure 5. Chlorophyll Concentration Index (CCI) of basil plants measured at 39, 49, 55, 62, 69, and 76 days after sowing (DAS), under different fertilization treatments. Values are means over two years. Error bars represent the standard error of means. Within each sampling day, different letters above the bars indicate statistically significant differences between treatments at $p < 0.05$.

TB production showed significant differences among the four fertilization regimes (Figure 6). Specifically, the FD-treated plants produced the highest TB, equal to 68.2 g plant^{-1}, while the F-treated plants followed. D-treated basil plants exhibited lower biomass production (59.3 g plant^{-1}), but significantly higher than the control. Unfertilized plants (C) presented the lowest biomass production (40.1 g plant^{-1}), approximately 41.2% less than the FD-treated plants and 32.4% less than the D-treated plants (Figures 6, 7).

Figure 6. Basil plants before harvest. From left to right: 1) unfertilized control plants; 2) fertilized with inorganic fertilizer; 3) fertilized with a combination of 50% nitrogen from digestate and 50% from inorganic fertilizer; 4) fertilized with digestate.

The FD-amended plants presented the highest total biomass compared to all other treatments, likely owing to the complementary effects of inorganic fertilization and digestate. Analogous results were reported by Adekiya et al. (2024), who observed enhanced maize production through the application of mineral urea combined with digestate. Other researchers have noted that substitution of mineral nitrogen fertilizers with digestate can increase fruit yield and plant growth considerably. In particular, Li et al. (2023) demonstrated that inorganic fertilization combined with digestate markedly increased tomato yield, compared to chemical fertilization. Jamison et al. (2021) reported enhanced plant growth in kai choy plants with 50% substitution of mineral fertilizers with digestate. Brychkova et al. (2024) found that partial or complete inorganic fertilizer substitution with digestate increased grassland quality and yield by 1.5 to 2-fold.

Plants amended solely with digestate exhibited significantly lower TB than F and FD plants, albeit performed considerably better (approximately 48%) than the control. These observations are in accordance with Ronga et al. (2018), who reported that digestate can serve as an effective alternative fertilizer for basil cultivation, especially in organic cultivation.

Hence, the markedly higher production of digestate-amended plants compared to the control highlights digestate's strong potential as a nutrient source for organic and sustainable cultivation systems, even though it may not serve as a complete nutrient source on its own.

Digestate is an untapped source of revenue for biogas plants (Kalaitzidis et al., 2026), as its usage as an alternative organic fertilizer would benefit the environment and enhance the biogas plants' profitability. According to Jurgutis et al. (2021), the average daily value of digestate produced from a 1 MW power plant can reach up to 1,518 €, providing a considerable source of income for biogas-producing units.

Nevertheless, there are certain constraints linked to its usage, such as low concentration of nutrients, elevated water content, considerable transportation and storage costs, and regulatory limitations (Martín-Sanz-Garrido et al., 2025c). Moreover, increases in pH, salinity, and Electric Conductivity (EC) (García-López et al., 2023), as well as the risk of pollution from heavy metals and pathogens (Derehajło et al.,2023; Lorine et al., 2021), pose a considerable concern, limiting its potential as a valid alternative to chemical fertilizers.

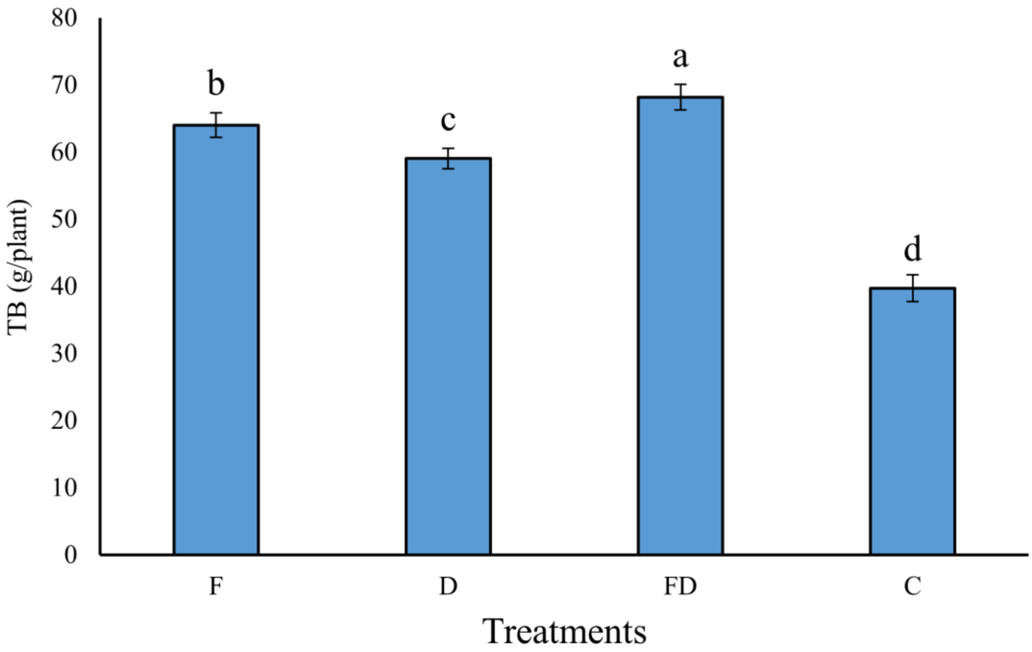

Figure 7. Total biomass (TB) of basil plants at 90 days after sowing (DAS), under different fertilization treatments. F: Inorganic fertilizer, D: Digestate, FD: Combined 50% inorganic fertilizer and 50% digestate, C: Control (no fertilization). Values are means over two years. Error bars represent the standard error of means. Different letters above the bars indicate statistically significant differences between treatments at $p < 0.05$.

Concerning the effect of the four fertilization regimes on the LP, our analysis showed that FD-treated plants exhibited significantly higher leaf production compared to all other treatments (Figure 8). Specifically, FD-treated plants produced 52.7 g plant^{-1}, while F-treated plants followed. In contrast, plants receiving solely digestate exhibited significantly lower LP, and unfertilized plants presented the lowest LP, equal to 28.9 g plant^{-1} (45% less than FD) (Figure 8).

The superior leaf production in FD-treated plants aligns with findings from other researchers who studied different basil species using combined mineral-organic fertilization strategies. Kalita et al. (2018) observed enhanced leaf yield with a combination of vermicompost and chemical fertilization (NPK) compared to the use of solely vermicompost in *O. gratissimum*. Likewise, Chandel et al. (2024) reported significantly higher numbers of leaves and leaf area in *O. gratissimum* through a combined inorganic-organic N fertilization. These results support our findings and highlight the synergistic action of organic and inorganic fertilizers.

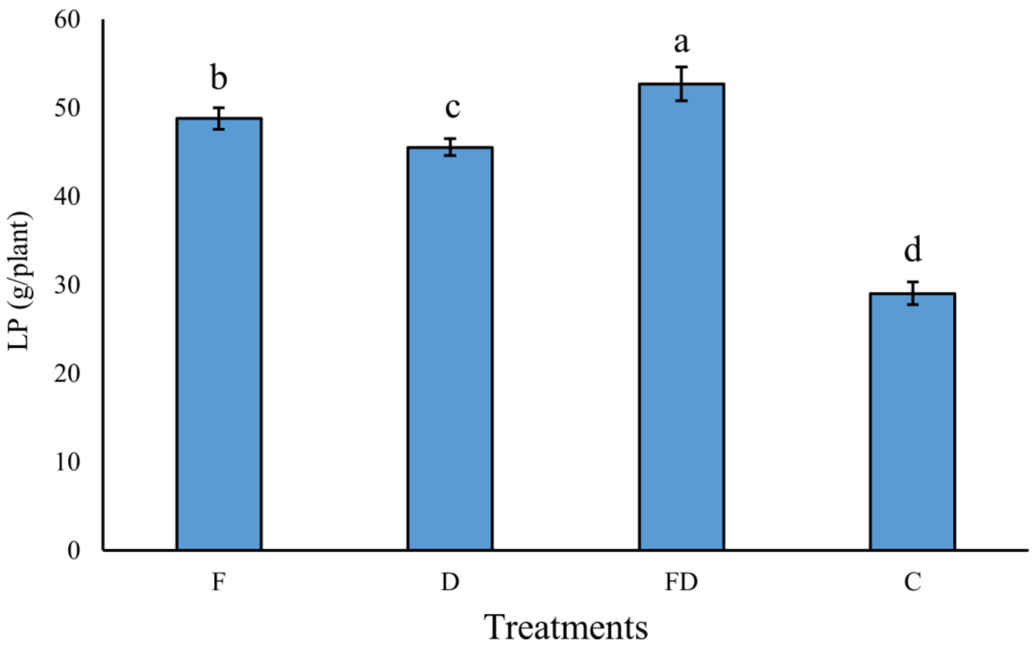

Figure 8. Leaf production (LP) of basil plants at 90 days after sowing (DAS), under different fertilization treatments. F: Inorganic fertilizer, D: Digestate, FD: Combined 50% inorganic fertilizer and 50% digestate, C: Control (no fertilization). Values are means over two years. Error bars represent the standard error of means. Different letters above the bars indicate statistically significant differences between treatments at $p < 0.05$.

3.2 Essential oil production and composition

Essential oil content in basil leaves was significantly affected by the different fertilization regimes. Specifically, all the fertilized samples exhibited lower EO content than the control (Figure 9). These results align with previous studies stating that excessive nitrogen fertilization leads to a reduction in essential oil production. Kordi et al. (2020) noted that the relationship between nitrogen fertilization and EO production is inversely proportional. Alami et al. (2024) reported that unfertilized plants produce more EOs to alleviate environmental and nutritional stresses. Hence, the high fertilization used in the current experiments led to reduced EO production in the leaves of the fertilized plants.

However, the FD-treated plants had the highest LP, while the control plants had the lowest. Therefore, if we estimate the essential oil yield of each treatment per hectare, we observe that the highest production is derived from the FD plants. Specifically, the FD leaves produced 24.77 kg ha^{-1}, the F 22.10 kg ha^{-1}, the D leaves 21.74 kg ha^{-1}, and the control 21.67 kg ha^{-1}. Thus, to maximize EO production, a combined fertilization is preferable.

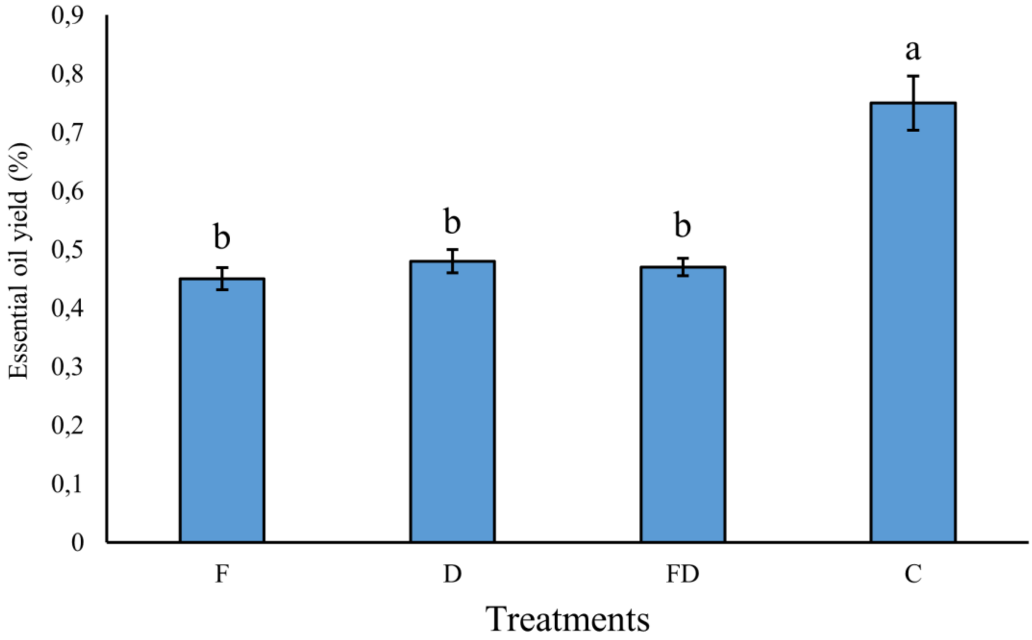

Figure 9. Essential oil yield (EO) of the basil plant leaves under different fertilization treatments. F: Inorganic fertilizer, D: Digestate, FD: Combined 50% inorganic fertilizer and 50% digestate, C: Control (no fertilization). Values are means over two years. Error bars represent the standard error of means. Different letters above the bars indicate statistically significant differences between treatments at $p < 0.05$.

The dominant constituent in the EO of the basil leaves, regardless of treatment, was linalool with concentrations ranging from 45.50 to 49.61%. The control plants demonstrated significantly higher linalool, limonene, and eugenol content, compared to all other treatments (Table 2).

The concentrations of linalool, limonene (monoterpenes), and eugenol (phenylpropanoid) are increased in plants to enhance their stress tolerance. These terpenes improve salt (Xu et al., 2024; Liu et al., 2025), cold and drought tolerance (Liu et al., 2025), and show excellent antibacterial, antifungal, antioxidant, and antineoplastic activity (Hosseini and Heidari, 2025; Liu et al., 2025). Therefore, the stress induced by the lack of nutrient availability likely facilitated the increase in these stress-alleviating components in the control plants.

Digestate fertilization ranked high in all sesquiterpenes and significantly increased the concentration of two. Specifically, D-treated plants exhibited the highest concentration in *β*-elemene and germacrene D (1.64 and 3.21%, respectively) (Table 2). Germacrene D is a volatile hydrocarbon and a key intermediate for the biosynthesis of several sesquiterpenes, including cadinenes (Barreto et al., 2021). Similar results were reported by Burducea et al. (2018), stating that basil fertilization with biosolids led to significant increases in the concentration of *β*-elemene and germacrene D. Ronga et al. (2018) reported enhanced sesquiterpene production in basil and peppermint plants grown hydroponically with solid digestate as the growing medium and liquid digestate as the nutrient solution.

There is no clear mechanism linking the synthesis of these sesquiterpenes with organic fertilization. However, the abundance of macro and micro-elements and O.M. in digestate, coupled with the presence of ammonium and nitrate nitrogen, has been known to influence the metabolic pathway of secondary metabolite synthesis (Ndah et al., 2022; Sile et al., 2022).

In contrast, plants receiving combined fertilization showed balanced results, exhibiting higher content in four monoterpenes (eucalyptol, trans-β-Ocimene, bornanone, and terpinen-4-ol) and two sesquiterpenes (α-bergamotene and α-humulene). Nevertheless, in most cases, the differences with the remaining fertilization treatments were not statistically significant.

Plants treated with mineral fertilizer exhibited significantly higher concentrations of methyleugenol and β-farnesene (3.47 and 1.31%, respectively). β-Farnesene is an important terpene with considerable and variable agricultural, pharmaceutical, cosmetic, bioenergy, and industrial applications (Rautela et al., 2024). Methyleugenol is a phenylpropanoid produced by plants as a defense mechanism for herbivores and pathogens and serves as a pollination attractant for certain insects, particularly fruit flies (Tan and Nishida, 2012). Hence, the terpene synthesis in F-treated plants showed that basil plants receiving inorganic fertilization focus on the synthesis of terpenes used for protection from biotic stresses and terpenes facilitating reproduction.

Notably, while F-treated plants showed the highest methyleugenol content, the control plants demonstrated the highest eugenol content. This inverse relationship suggests that under stress conditions, plants have limited methylation capacity for eugenol and, as a result, accumulate it. Conversely, under favorable nutritional conditions, plants exhibit enhanced methylation activity, converting eugenol to methyleugenol (Table 2).

Furthermore, germacrene D content was particularly low in F-amended plants and significantly higher in D-treated plants, suggesting that mineral fertilization could have an adverse effect on germacrene D synthesis.

Therefore, different fertilization regimes can stimulate the synthesis of different secondary metabolites. Fertilization with digestate enhances sesquiterpenes production and especially β-elemene and germacrene D; inorganic fertilization facilitates the production of methyleugenol and β-farnesene. Conversely, unfertilized plants produce leaves with higher linalool, limonene, and eugenol, while FD-treated plants achieve a more balanced profile with higher EO yield.

Table 2. Composition of the essential oil in the leaves of basil plants treated with four different fertilization regimes.

Peak number	Compound	RT	F	D	FD	C
1	Limonene	15.91	0.80b	0.80b	0.78b	0.86a
2	Eucalyptol	16.07	9.02a	7.43a	8.40a	7.41a
3	trans-β-Ocimene	17.34	2.10b	2.77a	2.67a	1.69c
4	Linalool	21.41	45.50b	46.86b	46.48b	49.61a
5	Bornanone	24.58	0.81a	0.74a	0.80a	0.64b
6	Terpinen-4-ol	27.45	3.75a	3.89a	3.67a	3.33b
7	Eugenol	42.23	6.13b	8.17b	7.41b	10.11a
8	β-Elemene	44.47	1.46b	1.64a	1.48b	1.49b
9	Methyleugenol	45.31	3.47a	2.05b	2.18b	1.93b
10	α-Bergamotene	47.05	6.29a	5.87a	5.56a	4.61a
11	α-Humulene	47.93	0.83a	0.80a	0.78a	0.61b
12	β-Farnesene	48.22	1.31a	1.02b	0.97b	0.92b
13	Germacrene D	49.40	2.31d	3.21a	2.59b	2.48c
14	τ-Cadinol	55.36	6.29b	7.57a	6.36b	7.18a
	Total		90.07	92.82	90.13	92.87

RT = Retention time. D: Digestate, F = Inorganic fertilizer, FD: Combined 50% inorganic fertilizer and 50% digestate, C: Control (no fertilization). Values are means over two years (*n = 6*). In each line, means with different letters indicate statistically significant differences between treatments at $p < 0.05$.

3.3 Soil properties

Soil pH in our study was similar in the F, FD, and D-treated plants, ranging from 7.7 to 7.8, representing a substantial reduction from the initial soil pH of 8.5. Conversely, the pH reduction in the control soil was limited and equal to 0.2 (pH = 8.3) (Table 3). These results differ from those typically reported in the literature, where digestate application generally increases soil pH. Specifically, according to García-López et al. (2023), digestate application in the cultivation of lettuce and kale resulted in considerable elevations in soil pH, significantly higher compared to the increases observed from soils fertilized with inorganic fertilizers. However, the usually high pH in digestates is beneficial to soils suffering from high acidity, because it increases their buffer capacity, protecting them from acidification, a major issue emanating from the constant addition of mineral fertilizers (Huang and Yu, 2022; Martínez et al., 2025).

In our study, soils amended with digestate showed a significant decrease in pH because the digestate's pH was adjusted to ~7.0 prior to application. In contrast, the pH in the control exhibited a minor reduction of approximately 0.2 units, likely due to the consistent addition of irrigation water (Table 3).

Salinity (S) and electrical conductivity (EC) in the soils of the D-treated plants were significantly higher compared to all other treatments (1.20 psu and 1.94 mS cm^{-1}, respectively). In contrast, soil from the control plants demonstrated the lowest S and EC values (0.16 psu and 0.22 mS cm^{-1}, respectively) (Table 3). These results align with García-López et al. (2023), who reported that increases in soil salinity were proportional to the addition of digestate. Salt content in digestate primarily derives from the substrates used for its production. The digestate used in the current study mainly comprised swine slurry, which has a high salt content (Marszałek et al., 2019). Moreover, rice straw was treated with Na, further increasing the digestate's sodium content.

Increases in soil EC over the threshold of 2.0 mS cm^{-1} are considered critical, and immediate action is required to avoid unwanted plant toxicity issues (Ragályi et al., 2025). Jamison et al. (2021) reported inhibitions in key growth and yield parameters in *Brassica juncea* plants when they used exclusively digestate from lignocellulosic biomasses for their fertilization, hypothesizing that the high EC of the media was responsible. However, proper management can alleviate any potential risks from digestate application. Ragályi et al. (2025) reported that adequate irrigation post-digestate application and digestate dilution prior to implementation efficiently mitigates salt accumulation toxicity phenomena and EC increases.

D-treated soils also showed significant concentrations of extractable phosphorus and boron, increasing the soil P and B content by 68.5 and 31.6%, respectively (Table 3). Conversely, samples from the control plants exhibited the lowest concentration, reducing P and B content by 20% and 10.5%, respectively. These increases in P availability with digestate application align with the research performed by Hammerschmiedt et al. (2022). The researchers reported that digestate application enhances soil P availability, particularly when different feedstocks are combined. García-López et al. (2023) stated that digestate affects nutrient availability and alters the biogeochemistry of phosphorus in soil, potentially increasing both inorganic and organic P fractions.

Calcium concentration in the examined samples showed that digestate application led to considerable elevations in soil Ca. Particularly, Ca levels in soils amended solely with digestate increased by 7.1%, while the FD soils presented the second-highest concentration. In contrast, samples from F and unfertilized plants exhibited the lowest Ca levels and actually removed Ca (approximately 7 and 8%, respectively) (Table 3). Other studies have reported similar results. Silva (2024) noted that digestate contains many essential nutrients, including calcium, which are mineralized and readily available for plant use.

Digestate application also resulted in significantly higher concentrations of iron, magnesium, manganese, sodium, and zinc. These results are on par with other researchers, stating that digestate is rich in minerals, and soils amended with digestate exhibit elevations in the concentration of nutrients significant for agricultural production. Moreover, according to Hammerschmiedt et al. (2022), digestate enhances the micronutrient content of soil, improves plant growth, and health. Rolka et al. (2024) reported that the addition of liquid digestate leads to considerable increases in K, P, Fe, Mn, Ca, Na, and total nitrogen, while the addition of solid digestate leads to major incremental changes in the concentration of available P, Mg, Mn, and exchangeable cations of Ca and Mg.

In contrast, FD and F samples presented the highest concentration of nitrate nitrogen, enhancing the soil content by 96 and 88.6%, respectively. Conversely, the increase in $N\text{-}NO_3$ from digestate application was significantly lower (69.6%), while the control reduced it by 52.1% (Table 3). According to the study of García-López et al. (2023), nitrogen in digestate is mostly organic, necessitating mineralization in order to become available to the plants. Hence, soils amended with inorganic fertilizers exhibited higher concentrations of $N\text{-}NO_3$, due to the immediate nitrogen availability from mineral fertilizers.

Soils amended exclusively with digestate presented a significantly higher concentration of organic matter (O.M.), increasing it by 54.7%, while FD soils followed. Conversely, soils from the F and control exhibited the lowest O.M., demonstrating limited increases equal to 2.8% and 1.7%, respectively (Table 3). The significant increases in O.M. from the use of digestate were expected, since several studies have supported this notion. In particular, Silva (2024) noted that soil amended with digestate presents considerable increases in organic matter, microbial activity, and nutrients. Villarino et al. (2025) reported that constant digestate application to 14 crop fields over more than five years led to markedly higher increases in soil organic carbon (SOC), particularly in soils with low initial SOC.

Concerning the K concentration, all the fertilized soils exhibited elevated K content, while the control exhibited reductions. Specifically, the highest concentration of K appeared in soils amended with digestate, elevating soil K content by 134.4%, significantly outperforming all other treatments. In contrast, the control soil exhibited the lowest concentration and reduced it by 8.1% (Table 3). Okoli et al. (2023) reported that digestate addition to soil enhances its potassium content. Yuan et al. (2023) stated that the combined application of inorganic and organic fertilizers leads to higher yields of sweet potato and enhanced K uptake, demonstrating digestates' ability to meaningfully elevate K concentration in soil.

Table 3. Soil chemical properties and nutrient concentrations under different fertilization treatments.

Chemical and nutrient properties	Treatments				
	Units	F	D	FD	C
pH	–	7.7a	7.8a	7.8a	8.3b
Salinity	psu	0.91c	1.20a	1.07b	0.16d
Electric Conductivity	mS cm^{-1}	1.30c	1.94a	1.47b	0.22d
^{E}Boron (B)	mg kg^{-1}	0.21c	0.25a	0.23b	0.17d
^{E}Calcium (Ca)	mg kg^{-1}	3373c	3868a	3720b	3332c
^{E}Phosphorus (P)	mg kg^{-1}	13.5c	20.0a	17.5b	9.5d
^{E}Iron (Fe)	mg kg^{-1}	5.25c	8.51a	7.04b	4.22d
^{E}Potassium (K)	mg kg^{-1}	380.0c	750.0a	650.0b	294.0d
^{E}Magnesium (Mg)	mg kg^{-1}	310.4c	420.6a	380.0b	274.3d
^{E}Manganese (Mn)	mg kg^{-1}	5.20c	6.70a	5.83b	3.38d
^{E}Sodium (Na)	mg kg^{-1}	135.1c	324.9a	271.7b	110.7d
^{E}Zinc (Zn)	mg kg^{-1}	2.84c	6.50a	4.80b	2.22d
Nitrate Nitrogen (N-NO3)	mg kg^{-1}	48.60a	43.70b	50.50a	12.35c
Organic Matter (O.M.)	%	1.86c	2.80a	2.53b	1.84c

E = Extractable. F: Inorganic fertilizer, D: Digestate, FD: Combined 50% inorganic fertilizer and 50% digestate, C: Control (no fertilization). Values are means over two years. Different letters indicate statistically significant differences between treatments at $p < 0.05$.

The increases in heavy metal concentration due to digestate addition were limited (Table 4). These findings align with the observations of Derehajło et al. (2023), who reported that digestate can contain variable trace amounts of heavy metals depending on the feedstock composition. Tang et al. (2020) reported that Cd levels in soils amended with digestate remain within safe limits after five years of constant application. In the study of Baldasso et al. (2023), researchers reported that digestate is safe to use for land restoration even if the concentration of heavy metals is high due to the low metal mobility in the soil profile.

Increases in Cu were limited, with the highest increases appearing in D-amended soils (Table 4). These results correspond with the findings of Ferreira et al. (2023), stating that pig slurry application increases the availability of the exchangeable fraction of copper in the soil without compromising crop productivity. Derehajło et al. (2023) reported that the digestate used in their experiments exhibited a higher concentration of mercury compared to animal slurry. However, Hg content was within the limits set for organic fertilizers (Regulation (EU) 2019/1009) and, therefore, safe for agricultural use. The researchers also stated that continuous monitoring of heavy metals is imperative to ensure their concentration remains within safe limits.

The authors of the current study concur that even though heavy metal content in the digestate and its translocation to the soil was limited, continuous monitoring is essential to ensure soil health and agricultural production free of heavy metal contamination.

Table 4. Soil heavy metal concentrations (mg kg^{-1}) under different fertilization treatments.

Heavy Metals	Treatments				
	Units	F	D	FD	C
^{T}Arsenic (As)	mg kg^{-1}	8.04a	8.18a	8.15a	7.85b
^{T}Cadmium (Cd)	mg kg^{-1}	0.19a	0.20a	0.19a	0.19a

^{T}Chromium (Cr)	mg kg^{-1}	75.29a	76.61a	76.12a	75.09a
^{T}Copper (Cu)	mg kg^{-1}	23.74a	24.13a	24.06a	23.47a
^{T}Lead (Pb)	mg kg^{-1}	15.1a	15.3a	15.0a	14.8a
^{T}Mercury (Hg)	mg kg^{-1}	0.05a	0.05a	0.04a	0.04a

T = Total. F: Inorganic fertilizer, D: Digestate, FD: Combined 50% inorganic fertilizer and 50% digestate, C: Control (no fertilization). Values are means over two years. Different letters indicate statistically significant differences between treatments at $p < 0.05$.

According to our pathogen contamination analysis, all samples were free of *Salmonella spp.* (Table 5). Low levels of *Enterococcus faecalis* and *Escherichia coli* were detected in the samples of the D-treated and FD-treated plants. However, their concentrations were very low (< 9.1 cfu g^{-1}). Conversely, these indicator organisms were not detected in the samples from the inorganic fertilizer or the control.

Furthermore, *Salmonella spp.* also remained undetected across all leaf samples. *Enterococcus faecalis* was found at estimated concentrations of 91 cfu g^{-1} and 73 cfu g^{-1} in the leaves of basil plants amended solely or partially with digestate (D and FD) (Table 5). This aligns with findings of Bonetta et al. (2014), who noted that properly processed digestate typically contains undetectable levels of *Salmonella spp.* Muhondwa (2019) reported a high survival rate of *E. coli* enteropathogens in mesophilic conditions, while Carraturo et al. (2022) noted that thermophilic anaerobic digestion of sewage sludge leads to complete *E. coli* pathogen elimination. Hence, anaerobic digestion in thermophilic conditions appears to be more efficient in eliminating indicator organisms, probably owing to elevated temperatures present during the thermophilic process, while mesophilic treatment appears unable to sufficiently sanitize the digestate.

These results indicate potential transfer of these bacteria either from soil to plant tissue or contamination during the application process. In contrast, *E. coli* was detected at very low levels (< 9.1 cfu g^{-1}) in leaf samples from digestate-amended treatments. Several studies have reported that indicator bacteria are able to survive anaerobic digestion, especially under mesophilic conditions (Lorine et al., 2021), while storage conditions also play a crucial role in mitigating pathogen survival and propagation (Schilling et al., 2022). Thus, it is safe to assume that improper digestate application and handling can contaminate plant matter and soil with pathogens, posing a risk for environmental and food safety.

The detectable concentrations of *Enterococcus faecalis* and *E. coli* on the leaves of the basil plants were below the levels of immediate concern for human consumption, but very close. Specifically, leaves from D and FD-amended plants exhibited 73 est. and 91 est. cfu g^{-1}, respectively. According to the EU, the threshold for ready-to-eat precut fruit and vegetables is 100 cfu g^{-1} (Commission Regulation (EC) No 2073/2005, 2020). These findings highlight the importance of appropriate post-harvest handling when digestate is used for edible crops. Similar conclusions were reached by other researchers, who emphasized the need for good agricultural practices when using organic fertilizers (Barra Caracciolo et al., 2022).

These results demonstrate that implementing appropriate post-harvest disinfecting protocols is imperative to minimize any potential contamination of plant tissues when digestate is used to fertilize leafy herbs and vegetables. Synchronously, the pasteurization of digestate as dictated by European law (Regulation (EU) 2019/1009) can significantly mitigate or even eliminate pathogens within digestate, while proper conditions of storage can further prevent risks of contamination (Schilling et al., 2022).

Table 5. Soil pathogen measurements under different fertilization treatments.

Pathogens	Treatments				
	Units	F	D	FD	C
	Soil				
Salmonella spp.	cfug^{-1}	N.D	N.D	N.D	N.D
Enterococcus faecalis	cfu g^{-1}	N.D	<9.1	<9.1	N.D
Escherichia coli (E. coli)	cfu g^{-1}	N.D	<9.1	<9.1	N.D
	Basil Leaves				
Salmonella spp.	cfu g^{-1}	N.D	N.D	N.D	N.D
Enterococcus faecalis	cfu g^{-1}	N.D	91est	73est	N.D
Escherichia coli (E. coli)	cfu g^{-1}	N.D	<9.1	<9.1	N.D

N.D: Not detected. F: Inorganic fertilizer, D: Digestate, FD: Combined 50% inorganic fertilizer and 50% digestate, C: Control (no fertilization).

4. Conclusions

This study demonstrated the efficiency of digestate as a source of nutrients for basil plant development, especially when combined with chemical fertilizers. The FD application was the optimum treatment, yielding significantly higher TB and LP, while sufficiently elevating the concentration of nutrients and organic matter (+54,7%) in the soil.

Furthermore, the use of solely digestate provided markedly higher values in all the metrics compared to the control. Hence, using digestate as fertilizer in organic basil production is worth exploring. Essential oil concentration in the control plants was significantly higher due to stress from limited nutrient availability. However, control plants exhibited superior linalool, limonene (monoterpenes), and eugenol (phenylpropanoid) content. This is particularly important because linalool was the main component of the EO, and eugenol content was almost double in the control plants compared to all the other treatments. In contrast, digestate significantly increased the concentration of sesquiterpenes, suggesting that specific fertilization strategies can influence secondary metabolite synthesis.

Additionally, the application of digestate did not significantly increase heavy metal concentration in the soil, showing that the digestate used in our study is safe to use as an organic fertilizer. Nevertheless, continuous monitoring of heavy metals in the digestate and soil is imperative to ensure soil health and food safety.

Finally, the considerable level of indicator organisms (*Enterococcus faecalis*) on the leaves of basil plants shows that precautionary measures, such as digest pasteurization and disinfecting protocols, must be implemented to ensure food safety.

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Chapter 6: General Discussion

The current thesis demonstrated that in order to use rice straw for biogas production, pretreatment and co-digestion are imperative. Initial rice straw pretreatment (p-RS) batch experiments showed increased biomethanation up to 15%, compared to untreated rice straw, while co-digestion with swine slurry (SS) enhanced methane production up to 59%, compared to SS mono-digestion. Our system, during the CSTR experiments, exhibited robustness under stress from high ammonia concentration and elevated OLR, showcasing that co-digestion leads to synergies that increase methane production and enhance the system's tolerability to stress.

Furthermore, our techno-economic analysis showed that the establishment of a biogas power plant in northern Greece treating p-RS and SS is economically viable with a payback period of 2.98 years and profitability similar to other biogas power plants in the Mediterranean region. However, profitability relied heavily on subsidized electricity prices and the low cost of raw material acquisition, while the addition of digestate sales significantly increased the profitability of the biogas power plant, highlighting the importance of making digestate a marketable product.

Our pot experiments with basil showcased that digestate is a valuable alternative to organic fertilizers and is worth exploiting. The 50% replacement of inorganic fertilizer with digestate resulted in the highest total biomass, leaf production, and essential oil production per hectare. Essential oil composition was also influenced by digestate addition, with sesquiterpenes being significantly affected, in accordance with other researchers (Ronga et al., 2018). These results demonstrate that digestate is a valuable fertilizer alternative that closes the nutrient loop, transforming the biogas system into a biorefinery system where outputs from one process become valuable inputs for the next.

The increases observed in rice straw biomethanation due to the thermochemical pretreatments primarily stem from the ability of alkalis to cleave the ester-ether bonds linking lignin to polysaccharides, while weakening hydrogen bonds between hemicellulose and cellulose. In our experiments, the optimum pretreatment involved the use of NaOH (6% TS w/w) at 55 °C for 24 hours (THEC1). This thermochemical approach achieved substantially greater methane yields than thermal pretreatment alone (limited 2-5% improvements), consistent with other researchers who reported improved lignocellulosic degradability with alkali pretreatment (Shakeel et al., 2024, 2022). Moreover, the use of mild temperatures (55 °C) prevented the formation of inhibitory substances such as furfural and 5-HMF, which can be formed during pretreatment at high temperatures (Ahmed et al., 2022; Balasundaram et al., 2022).

However, the high C/N ratio in lignocellulosic substrates necessitates the co-digestion with a nitrogen-rich co-substrate. Therefore, the co-digestion of p-RS with swine slurry at a 50%:50% VS ratio was advantageous, yielding a significant increase (59%) compared to the mono-digestion of swine slurry. Co-digestion with cattle slurry also improved methane production by up to 14%. However, the high nitrogen content and the synergistic effects stemming from the co-digestion of p-RS with SS proved superior. Similar results were reported by other researchers who examined the co-digestion of swine slurry with rice straw (Tian et al., 2023a; Zhong et al., 2021). Co-digestion is more advantageous than the mono-digestion of livestock manures because it dilutes toxic substances, such as ammonia, improves feedstock's moisture content, enhances nutrient availability and diversification, and increases the buffer capacity of the feedstock (Kadam et al., 2024b). Additionally, it enhances microbial and enzymatic diversification and promotes synergies, leading to higher substrate biodegradation (Sudiarto et al., 2025).

The buffer capacity and improved robustness of the system were highlighted in the CSTR experiments, where high OLRs and ammonia accumulations disrupted the system's stability. However, it did not collapse but rather reached a new equilibrium without process failure. Furthermore, gradual increases from 2 gVS L^{-1} d^{-1} to 3 gVS L^{-1} d^{-1} and then to 4 gVS L^{-1} d^{-1} resulted in relatively quick system adaptation, with limited VFA accumulation (~150 mg L^{-1}) and sustained methane yields of 348-353 mL CH_4 gVS^{-1}. In contrast, instantaneous increases from 2 gVS L^{-1} d^{-1} to 4 gVS L^{-1} d^{-1} and then to 6 gVS L^{-1} d^{-1} resulted in a significant methane reduction (331 mL CH_4 gVS^{-1}) and an increase in CO_2 production. This was probably due to a shift in the methanogenic pathway from acetoclastic to hydrogenotrophic (Bucci et al., 2024). Several researchers have reported that gradual increases in OLR are more advantageous than instantaneous increases, because the microbial community acclimates more easily (Ahmad et al., 2021; Ketheesan and Stuckey, 2015). Instantaneous increase in the OLR of R3 (from 4 to 6 gVS L^{-1} d^{-1}) resulted in a significant decrease in the percentage of methane in the biogas mixture (51%), compared to 64-65% in R1 and R2. The recovery of the system was estimated to be ~ 30 days, after which it continued to operate under lower methane yields and increased ammonia concentrations.

Specifically, the system tolerated TAN increases of approximately 3,500 mg L^{-1} without complete failure, exceeding the typical inhibition threshold of 1,000 to 3,000 mg L^{-1}. Free ammonia concentrations reached 212 mg L^{-1} in R3, surpassing the 200 mg L^{-1} threshold associated with process disruption. The dilution of toxic compounds and the synergistic effects of co-digestion probably mitigated the adverse effects of excessive TAN and FE concentrations in R3 and prevented the collapse of the process. Additionally, the strong and negative correlation between TAN and methane yield ($r = -0.826$) confirmed ammonia's inhibitory role, highlighting the robustness of the system and underscoring the practical applicability of this co-digestion approach for commercial implementation. The system's robustness was also demonstrated by the chemical oxygen demand (COD) removal efficiency. COD removal efficiency remained consistently high across all reactors during the first period (65.7-65.9%), with gradual improvements to 67.9% in R1 and 66.6% in R2 by period III. These values align with literature reporting 53-70% COD removal for rice straw and swine manure co-digestion systems. Notably, R3 maintained a 65.3% efficiency during period II but declined to 62.4% in period III, demonstrating that while the system avoided complete failure, stress conditions did impair organic matter degradation capacity. The relatively modest decline (5.5% less than R1) despite TAN approaching 3,500 mg L^{-1} and free ammonia exceeding 200 mg L^{-1} further underscores the system's resilience. Our co-digestion system maintained acceptable COD removal even under critical stress conditions, supporting the practical feasibility of commercial operation where occasional process disruptions are inevitable.

The potential of digestate as an alternative fertilizer for MAP cultivation was highlighted in our basil pot experiment. Plants receiving 50% of their nitrogen needs from digestate and 50% from inorganic fertilizers presented significantly higher total biomass, superior leaf production, and essential oil yield per hectare, even though control plants exhibited higher essential oil production per 100g of fresh leaves.

Moreover, control plants produced significantly higher essential oil components associated with stress, such as linalool (49.61%), limonene, and eugenol, while fertilized plants exhibited higher terpenes related to aroma, flavouring, herbivore repellent, and attraction of pollinators. Plants receiving solely digestate fertilization exhibited significantly elevated sesquiterpenes, particularly germacrene D (3.21%) and *β*-elemene (1.64%). However, the mechanism linking organic fertilization to enhanced sesquiterpene biosynthesis remains unclear, though the abundant macro and micronutrients in digestate, coupled with the presence of both ammonium and nitrate nitrogen, likely influenced secondary metabolite pathways differently than solely mineral nitrogen fertilization (Ndah et al., 2022; Sile et al., 2022). In contrast, mineral fertilization favored methyleugenol synthesis (3.47%), while the combined fertilization approach presented a more balanced terpene profile with moderate concentrations across multiple compounds. Such differences in terpene synthesis have potential commercial applications since targeted fertilization appears to affect secondary metabolite synthesis. Specifically, no fertilization leads to the production of monoterpenes and especially linalool, digestate fertilization enhances sesquiterpene synthesis and especially germacrene-D, combined fertilization leads to a more balanced profile, while mineral fertilization favors the production of methyleugenol.

Notably, digestate addition did not result in significant increases in soil heavy metal content. Probably because the digestate used in our study was very low in heavy metals, and as a result, translocation to the soil was minimal. Furthermore, the concentration of all macro and micronutrients increased significantly, except nitrogen, which was particularly high in soils amended partially or entirely with inorganic fertilizers. Specifically, the concentration of macronutrients K and P increased by 134.4 and 68.5%, respectively, compared to their concentration in the soil prior to experimentation, while micronutrients such as Mg, Mn, Fe, Zn, and B also showed significant increases.

EC and soil salinity were high in soils amended with digestate (1.94 mS cm^{-1} and 1.20 psu, respectively), but remained marginally below the threshold for immediate action (2.0 mS cm^{-1}). The naturally high salinity of SS (Marszałek et al., 2019), coupled with rice straw's pretreatment with NaOH, probably resulted in enhanced sodium accumulation and increased EC. Our digestate adjustment to ~7.0 prior to implementation lowered soil pH and potentially facilitated better nutrient uptake, as most plants require neutral to slightly acidic soil for enhanced nutrient absorption (Barrow and Hartemink, 2023).

Crucially, *Salmonella* pathogens were not detected either in the soil or on the leaves of the basil plants. However, indicator organisms *E. coli* and *Enterococcus faecalis* were present on both the soil and the leaves. Specifically, both indicator organisms were below 9.1 cfu g^{-1} and within safety limits in the soil. *Enterococcus faecalis* was detected on basil leaves at 91 cfu g^{-1} (D treatment) and 73 cfu g^{-1} (FD treatment). These concentrations are marginally below the EU safety limit of 100 cfu g^{-1} for ready-to-eat fruit and leafy vegetables. Nevertheless, *E. coli* remained at very low levels on leaves (< 9.1 cfu g^{-1}). These findings align with literature stating that mesophilic anaerobic digestion (37 °C) provides insufficient inactivation of indicator organisms. Studies consistently show that thermophilic digestion (55-60 °C) achieves superior pathogen elimination due to sustained elevated temperatures, while mesophilic processes allow certain enteric bacteria to survive (Carraturo et al., 2022).

Therefore, treating digestate prior to implementation is imperative to avoid unwanted translocation and contamination from enteric pathogens. According to EU Regulation 2019/1009, pasteurization of digestate and other organic fertilizers prior to use is compulsory to avoid pathogen proliferation. For leafy herbs and vegetables, implementing post-harvest disinfection protocols provides an additional safety measure, as proposed by Dhull et al. (2024). Thus, digestate can be used successfully for conventional or organic cultivation, but proper management prior to implementation and post-harvest is imperative to ensure food safety and enhanced soil health.

The techno-economic analysis revealed that the construction of a biogas power plant in northern Greece treating p-RS and SS is a viable scenario, with profits similar to other plants situated in the Mediterranean region. The return on investment was estimated at 33.54% $year^{-1}$ and the payback period at 2.98 years, suggesting that the economic outlook of the project is highly favorable. The system would process 34,058 tons of agricultural waste annually and generate 15,891 MWh $year^{-1}$ of renewable energy. Moreover, all of the substrates would be provided by local farms, thus sustainably treating local agricultural waste and creating job opportunities for the local rural population. However, profitability mainly depended upon governmental subsidies (€225 per MWh under Greek legislation) and low feedstock acquisition costs. Therefore, fluctuations in electricity prices, governmental subsidies, and transportation and handling costs of the substrates could significantly undermine the project.

Notably, the addition of digestate sales considerably increased the plant's profitability and elevated it from €955,358 to €1,509,428, increasing net profit by 58%. Hence, digestate can significantly increase a plant's profitability and viability, making it less susceptible to fluctuations in subsidies and feedstock costs.

Thus, this thesis advances the understanding of agricultural biogas systems by demonstrating that technical optimization, economic viability, and environmental sustainability require integrated management across the entire value chain. Sustainable rice straw valorization depends on effective pretreatment and co-digestion with a co-substrate that enhances both methane production and process stability. Additionally, the use of digestate as an alternative fertilizer closes the nutrient loop and can generate invaluable revenue for the biogas power plants. The 59% improvement in methane yield, combined with the system's robustness under ammonia stress and elevated organic load, shows that practical application of our co-digestion mixture is feasible. The 2.98-year payback period demonstrates economic viability, but requires digestate commercialization. The successful application of digestate as a partial source of nutrients for basil development validates our circular biorefinery concept. These findings provide a roadmap for Mediterranean agricultural regions seeking to convert agricultural residues into renewable energy and valuable soil amendments, transforming waste into opportunity while advancing climate mitigation and agricultural sustainability goals.

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Chapter 7: General Conclusions

This study highlighted that anaerobic digestion of rice straw, through pretreatment process optimization and co-digestion with livestock slurries, is an excellent substrate for biogas production and sustainable management of agricultural residues. CSTR experiments showed that our co-digestion system is robust and can operate successfully under severe stress from ammonia accumulation and instantaneous increases in the organic load. The techno-economic analysis revealed that making digestate a marketable product can substantially increase the profitability of the biogas power plants, up to 58%. Additionally, the use of digestate as an alternative fertilizer for basil development revealed that, coupled with mineral fertilization, it is superior to solely mineral fertilization, while digestate alone is a promising fertilizer for basil organic cultivation. Moreover, digestate addition increased sesquiterpenes synthesis and nutrient availability in the soil and did not enhance heavy metal content in the soil. The presence of indicator organisms on the leaves of basil post-harvest suggests that the pasteurization of digestate and disinfection protocols are mandatory to ensure food safety and soil health.

The key points of our research are:

- Thermochemical pretreatment of rice straw with NaOH at mild temperatures (55 °C) can significantly increase rice straw biodegradability (up to 15%) without the risk of forming inhibitory compounds such as furfural and 5-HMF. Thermal pretreatment results in limited increases up to 5%. The ability of alkalis to effectively disrupt the lignin-polysaccharide bonds and reduce cellulose crystallinity probably led to higher rice straw biodegradation.

- Co-digestion with livestock slurries improved substrate biomethanation. Specifically, co-digestion of pretreated rice straw (p-RS) with cattle slurry (CS) increased methane production up to 14%, while co-digestion with swine slurry (SS) up to 59%, compared to the mono-digestion of each slurry. The improved C:N ratio, higher nutrient availability, the presence of hydrolytic enzymes in swine slurry, and the synergistic effects stemming from the co-digestion of SS and p-RS significantly improved the substrates' biodegradation and biomethanation potential.

- The techno-economic analysis revealed that the establishment of a biogas power plant in northern Greece is a feasible and profitable scenario. The plant will generate 15,891 MWh year^{-1} of renewable energy and sustainably treat 34,058 tons of waste. Net profit can reach €1,510,000, the payback period can be as low as 2.98 years, while return on investment and profit margin can be as high as 33.5% and 62.4%, respectively, including the earnings from digestate sales. Without digestate sales, the net profit decreases to €955,358, the payback period increases to 4.71 years, while return on investment and profit margin decrease to 21.23% and 51.26% per year, respectively. However, profitability relies heavily on subsidies for electricity prices and low acquisition costs for substrates. Therefore, digestate sales can meaningfully increase the profitability of biogas power plants (up to 58%), making them less susceptible to fluctuations in electricity prices and substrate acquisition costs.

- The CSTR experiments showed that co-digestion of p-RS with SS (70%:30% VS) can yield up to 353 ml CH_4 g VS^{-1}, with a 65.6% methane content. The system tolerated significant stresses from elevated organic loads and ammonia accumulation, with pH remaining within a high yet acceptable range (~7.4–8.0). Gradual and especially instantaneous increases in OLR led to considerable increases in volatile fatty acids (VFAs), with the system always recovering, typically within ~30 days. Severe stress from high ammonia accumulation (TAN = ~3,500 mg L^{-1}, FE = 212 mg L^{-1}) did not cause process failure but led to a new equilibrium at lower levels (331 mL CH_4 gVS^{-1}, 51% methane). The inhibitory role of ammonia was affirmed through a strong and negative correlation (r = –0.826) between TAN and methane production. Notably, chemical oxygen demand (COD) removal remained stable (65–67%), except in R3 during the third period, when COD removal efficiency dropped to 62.4%. However, the 5.5% reduction, compared to the highest removal rate, was relatively low considering that TAN and FE levels were ~3,500 mg L^{-1} and 212 mg L^{-1}, respectively, exceeded the thresholds of 3,000 mg L^{-1} and 200 mg L^{-1} for severe process disruption. Thus, the system demonstrated robustness, which has important practical implications since, in practice, process disruptions are inevitable.

- Our study on the use of digestate as an alternative organic fertilizer for basil development revealed that digestate combined with chemical fertilizers in a 50%:50% nitrogen ratio is superior to solely mineral fertilization in terms of total biomass production (TB), leaf production (LF), and essential oil yield production per hectare. Moreover, it significantly increased the nutrient content of the soil and the organic matter (54,7% increase). Notably, plants treated exclusively with digestate exhibited significantly higher metrics compared to the control plants, highlighting digestate's potential as an effective alternative for organic basil production. Control plants exhibited higher essential oil yield, but combined fertilization resulted in higher essential oil production per hectare. Digestate addition promoted sesquiterpene synthesis, mineral fertilization favored the production of terpenes associated with biotic stress and plant pollination, while the absence of fertilization resulted in enhanced synthesis of terpenes associated with abiotic stresses. Therefore, it appears that different fertilization regimes can lead to the synthesis of different terpenes, having potential industrial applications. Importantly, digestate application did not increase the accumulation of heavy metals in the soil, while the appearance of high (yet within permissible levels) of *Enterococcus faecalis* suggests that pasteurization and disinfection protocols are imperative to ensure food safety and soil health.

- Future research should include a life cycle assessment (LCA) analysis to quantify the full environmental benefits of this integrated system, including greenhouse gas emission reductions and fossil fuel displacement. Furthermore, investigating hydrolysis kinetics and startup time could elucidate the mechanisms enhancing methane production from co-digestion versus mono-digestion, facilitating further process optimization.

In conclusion, our biorefinery system approach appears feasible and promising. Proper pretreatment and co-digestion of rice straw leads to high methane yields, while the system exhibits superior robustness to disruptions, inevitable in a real-world scenario. Moreover, digestate use, especially combined with conventional fertilizers, results in superior basil plant yields while significantly improving soil properties. Moreover, the presence of indicator organisms on the leaves of basil highlights the need for digestate pasteurization and the use of disinfection protocols post-harvest. Finally, we uncovered that different fertilization regimes appear to influence secondary metabolite synthesis, which can be an important asset for industrial use.

Appendices

Figure 1. Storage of rice biomass in a freezer at a temperature of -20°C.

Figure 2. Laboratory mill used for milling the rice biomass with a 0.5 mm sieve

Figure 3. Crucibles placed in a muffle furnace to estimate volatile solids (VS).

Figure 4. Portable laboratory desiccator where samples were placed after being removed from the muffle furnace.

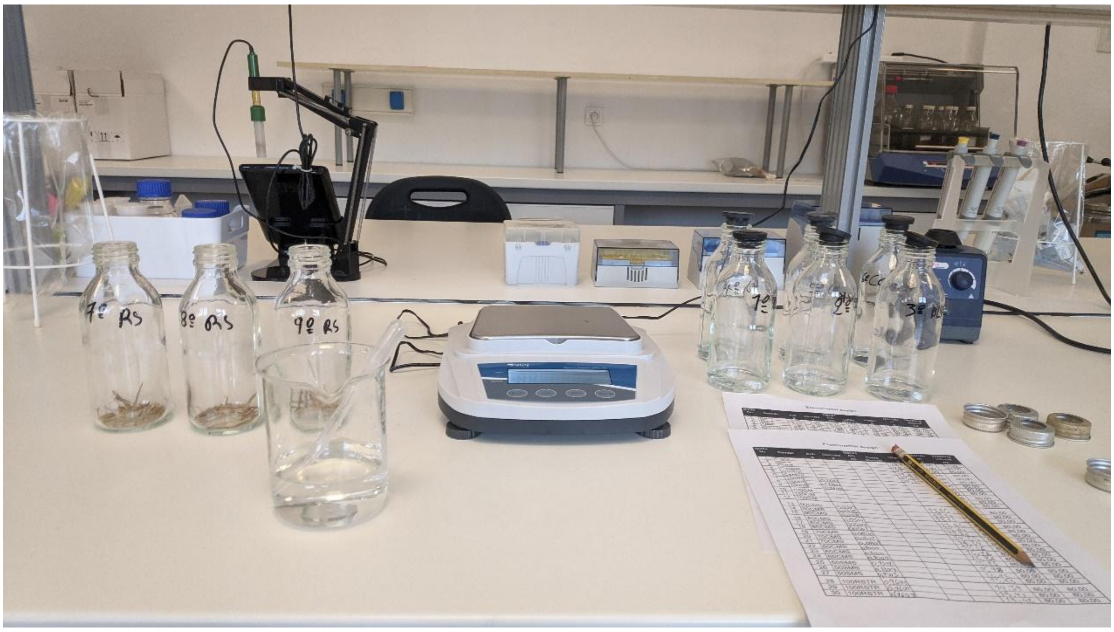

Figure 5. Glass reactors and substrate used in the pretreatment experiments

Figure 6. Glass reactors used in the co-digestion experiments

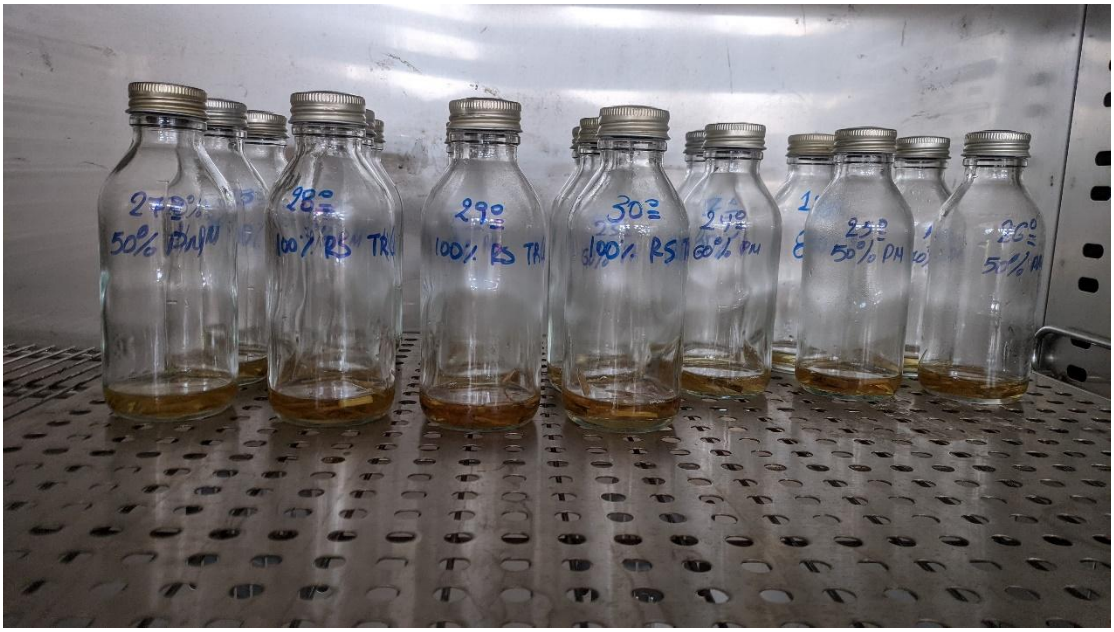

Figure 7. Glass reactors placed in the incubator for substrate pretreatment prior to the co-digestion experiments

Figure 8. Glass reactors removed from the incubator after substrate pretreatment and prior to the co-digestion experiments

Figure 9. Basil plants have two fully developed leaves.

Figure 10. The methodology employed to sieve the soil with a 2 mm sieve.

Figure 11. Sieved soil and perlite in a 2:1 (v/v) ratio. A plastic tray with young basil plants ready for transplanting is also visible in the center of the figure.

Figure 12. Basil plants at 60 DAS. Fertilized with inorganic fertilizer (left), with digestate (center), with a combination of 50% nitrogen from digestate and 50% from inorganic fertilizer (right).

Figure 13. Basil plants at 60 DAS. From left to right: 1) unfertilized control plants; 2) fertilized with inorganic fertilizer; 3) fertilized with a combination of 50% nitrogen from digestate and 50% from inorganic fertilizer; 4) fertilized with digestate.

Chapter 8: Scientific Outputs

Sections of the current thesis have been published in two (2) peer-reviewed scientific journals and in two (2) international poster sessions. In addition, one (1) more publication is in the printing stage.

8.1 Published and "Under review" manuscripts

Kalaitzidis A, Mitraka G-C, Katsantonis D, Prasad S, Singh A, Koutroubas S, Kougias PG and Korres NE (2026). Enhancing methane production from rice crop residues via pretreatment and co-digestion with cattle or swine slurry. Front. Energy Res. 13:1680055. doi: 10.3389/fenrg.2025.1680055.

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8.2 Poster Presentation

A. Kalaitzidis, N.E Korres, G.-C. Mitraka, D. Katsantonis, P.G Kougias. Utilization of rice crop residuals for biogas production through anaerobic digestion. 10^{th} International Conference on Sustainable Solid Waste Management, 21-24 June 2023, Chania, Crete, Greece.

Argyrios Kalaitzidis, Panagiotis Kougias, Nicholas E. Korres, Georgia-Christina Mitraka, Anastasios Mitsopoulos, Sofia Zioupou, Charalampos Petkoglou, and Dimitrios Katsantonis. Pre-treatment of rice crop residues to enhance biogas production process. 17^{th} International Conference of the Hellenic Association of Agricultural Economists (ETAGRO 2023).