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EFFECT of digestates derived from the fermentation of maize-legume intercropped culture and maize monoculture application on soil properties and plant biomass production

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Abstract

Background: The use of maize-legume mixed culture to produce renewable energy and fertilizers by anaerobic fermentation (AD), while respecting soil quality is a favourable approach in sustainable farming. This paper investigates how the substrate (silage) composition affects the quality of digestate and thus its effect on selected soil parameters (respiration, content of carbon and nitrogen). The high content of remaining nutrients (mainly N) in the AD residual biomass of digestate may increase the biomass of amended plants. One objective of this study was to determine the composition of different digestates produced by anaerobic fermentation of the biomass of intercropped (mixed) cultures. Other objectives focused the digestate impact on soil properties and yield of tested plant (lettuce) in a pot experiment, carried out under controlled conditions in the growth chamber for 6 weeks. Variants tested in the pot experiment included negative control, maize (*Zea mays* L.) digestate, broad bean (*Vicia faba* L.) digestate, white lupine (*Lupinus albus* L.) digestate, maize + broad bean digestate, maize + white lupine digestate.

Results: As compared to maize, silage from the mixed culture (or legumes) positively affected the properties of digestate (content of N, P, K, Acid Detergent Fibre (ADF), Neutral Detergent Fibre (NDF), Acid Detergent Lignin (ADL). The effect of digestate application on soil parameters depended on the digestate composition: the highest basal respiration was induced by digestates with the increased content of dry matter and ADF – maize + broad bean and white lupine. The broad bean variant showed glucose-induced respiration 0.75 ($\mu g\ CO_2 \cdot g^{-1}\ h^{-1}$), while the lowest value was in the maize variant ($0.45\ \mu g\ CO_2 \cdot g^{-1}\ h^{-1}$). The application of digestate derived from the mixed culture increased the plant biomass more than that of single maize silage digestate (+ 14% in the maize + broad bean variant and + 33% in the maize + white lupine variant).

Conclusions: A potential was found of silage made of leguminous plants to increase the digestate N content. Nevertheless, it is desirable to increase the C/N ratio by raising the amount of C containing substances. Fertilization with digestate showed a potential to increase the plant biomass (compared to the unfertilized control); however,

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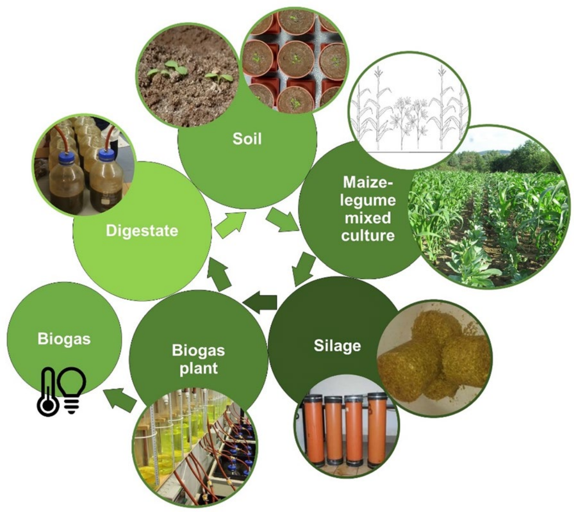
differences among the individual digestates were not observed. The benefit of legume added to the maize-based silage was proven, especially the contribution of nutrients to arable soil.

Keywords: Soil fertility, Microbial activity, Carbon, Nitrogen, Sustainable agriculture

Graphical abstract



Introduction

Rapid urbanization and industrialization in the last decades have significantly changed land use and land cover of traditional agricultural areas worldwide. The subsequent agricultural intensification has increased needs for inorganic fertilization in agricultural management, which affects soil quality as well as health and crop productivity. In the last years, the concern about environmental problems caused by the excessive use of chemical fertilizers is rising. The adoption of agricultural practices that conserve soil organic carbon can increase food production. One of approaches is a long-lasting application of organic amendments. Depending on the type of the applied organic amendment, different changes in soil properties and fertility were observed in a broad time horizon under different pedoclimatic conditions [1]. Farmyard fertilizers produced as waste materials of livestock production such as manure and slurry were the most beneficial. However, due to the markable impact of livestock production on the global emissions of greenhouse gases, other strategies for utilizing other types of waste-based organic fertilizers such as digestate are intensively sought.

Anaerobic fermentation is a technological process generating two products: biogas mainly composed of methane which is subsequently used as a renewable energy source, and digestate, a potential fertilizer and or soil amendment. Digestate is an organic material with characteristic agronomic properties that make it suitable for the application on agricultural lands [2, 3]. Digestate is rich in carbon (C), nitrogen (N), phosphorus (P), potassium (K), sulphur (S), calcium (Ca), magnesium (Mg), and microelements. The use of digestate in agriculture has been shown to have positive effects on the crop growth and production [4]; thus, its use is commonly recommended. Processes happening during the anaerobic fermentation of organic materials (feedstock) improve properties of biomass source, e.g., enhance the availability of N (mainly in the NH_4^+ form) [5] and P [6] to plants. Due to these features, the digestate-based fertilization can be equally efficient as conventional organic amendments, e.g., farmyard manure or compost. It was reported that the addition of digested exogenous organic matter (EOM) to the soil led to C sequestration due to inherent stability of EOM in comparison to the undigested-EOM addition [7]. Unlike the traditional soil amendments, organic manures, sewage sludge and composts which have been extensively studied hitherto [8, 9], research regarding the application of digestates is far its full capacity. It was reported that the application of digestate as compared to manure lead to a significantly greater yield of hay [10]. Therefore, many questions regarding the applicability of digestate to improve physical, chemical, and biological soil properties need to be unveiled and thus require further research.

Important sources for biogas production are energy crops, such as maize and beets [11]. In Europe, maize (*Zea mays* L.) is a crop used most in the production of biogas and digestates [12, 13]. To increase the energy potential of maize or efficiency and sustainability of biomass production, different approaches have been tested and applied, e.g., (1) optimization of mono-fermentation processes [14], (2) optioning of particular grown hybrid and harvest at a certain stage of ripeness [15], and (3) the use of mixed cultures, such as maize and intercropped sunflower [16], sorghum [17] or legumes [18]. Testing of anaerobic co-fermentation was also underway in recent years. This, in contrast to mixed culture, is devoted to the separate cultivation of plants and a subsequent process of fermentation of the mixture of two or more substrates. This process helps to increase biogas production and to stabilize the entire fermentation process. However, this technology did not alleviate the negative effect of maize mono-cultivation on agricultural land leading to decreased resilience to soil erosion [19].

Mixed culture is currently defined as growing two different crops on the same plot at the same time [20]. The reason for changes was the potentially adverse impact of conventional maize cultivation technology and doubts, whether and to what extent the growing of maize only for biogas production is ecological [21, 22]. Maize cultivation technology based on intercropping (intra-row or inter-row) is a sustainable agricultural practice [18]: it mitigates negative impacts on the environment and the use of biomass from mixed cultures is accompanied by higher biogas yield benefits [22]. So far, the primary interest in the improvement of various intercropped biomass producing technologies was preparation of optimal raw material, i.e., finding a mixture ratio balanced for agricultural systems and biogas technology [23] and increasing the biomass and biogas yield [24]. Until now, only few studies were focused on the impact of mixed culture type on the residual N content in the produced digestate and comparative fertilization performance of digestate and mineral fertilizer [25]. Mineral fertilizer (calcium ammonium nitrate) and a combination of liquid digestate fraction + mineral fertilizer mostly led to the highest quantitative biomass yields at the equal N dose applied per hectare. Moreover, the application of digestate obtained from the anaerobic fermentation of intercropped mixed cereal and legume biomass has a potential to supply soil and plants with nutrients more efficiently as compared to the intercropped biomass of triticale and clover grass in the respective study by Ehrmann et al. [25].

The main advantage of intercropping cereals and legumes is that legumes contribute by providing N sources to non-legumes. This increases biomass production per unit area in comparison with monoculture cropping due to the efficient use of resources including water, nutrients, and solar energy [26]. However, it was observed that a higher content of ammonium or coumarin from legumes in the intercrop biomass could inhibit the production of biogas during fermentation [27–29]. As the system of cereals and legumes culture is an important approach in sustainable farming systems [30] and in the use of renewable resources [31], the complete most beneficial utilization of both the sources and the end products of the respective technological process is still required. The high content of nutrients (mainly N) remaining in the residual biomass of different variants of digestate may increase the harvestable biomass of amended plants constantly with the increasing N-rate [32]. The N-rich digestate amendment to non-legumes increased the N_2 fixation compared to biomass left in situ [33]. Therefore, the storage of biomass for reallocation (in the form of digestate) in spring may lead to the accumulation of N in digestate-amended systems. A consistent recycling of the residual biomass via amendment to the soil has a potential to enhance the subsequent crop biomass productivity [34].

The objective of this study was to determine the impact of digestates obtained as residues from biogas produced by anaerobic fermentation of mixed maize and legume culture silages, on the soil properties and tested plant yield. Different types of digestates produced by anaerobic fermentation of biomass from diverse mixed crop cultures varied in their chemical parameters: dry matter and N-compounds content. Amendment of 100 kg N per hectare in the form of different variants of digestate was compared and the effect on biological properties and nutrient content in the soil and on the yield of lettuce (*Lactuca sativa* L.) was evaluated. We hypothesized that (1) silage from the mixed culture (or from the *legume*) would positively affect the properties of digestate produced during the anaerobic fermentation, (2) application of individual digestate types would have a variable effect on selected soil parameters and soil fertility (depending on the N-content and different availability of nutrients, organic matter and microbial community of each digestate type), (3) the application of digestate would have a positive effect on the yield of cultivated crop compared to the control variant.

Materials and methods

Experimental field, plant biomass and preparation of silages

The plant biomass intended to produce biogas and the subsequent fermentation of residues was grown at the Experimental Station for Fodder Crops in Vatín. Further details on the biomass cultivation can be found elsewhere

[35]. The station is located approximately 7 km from Žďár nad Sázavou in the Czech Republic in a moderately warm area of the Bohemian-Moravian Highlands with a long-term average annual temperature of 7 °C and a year-round long-term average rainfall of 658.6 mm, the values correspond with climate standard in 1981–2010. The soil of this site is characterized as Cambisol sandy loam. The following variants were grown: (a) monoculture of maize (*Zea mays* L.)—M; (b) monoculture of broad bean (*Vicia faba* L.)—B; (c) monoculture of white lupine (*Lupinus albus* L.)—L; (d) mixed culture of maize and broad bean—MB; (e) mixed culture of maize and white lupine—ML. Each of the above-mentioned variants was sown into a belt (w 6 m, l 50 m) with a buffer zone (w 1.5 m) being left on each side. Thus, all variants of the experiment had an area of 180 m^2. Each variant had three replicates, and randomization was used to prevent bias results.

The experiment was established using a Kinze 3500 seed drill—the "interplant" model. Sowing was performed by alternating 2 rows of maize with two rows of legumes (Fig. 1) to achieve a mixed culture system. The sowing dose was 75 thousand seeds of maize (*Zea mays* L.—FAO 270) and 75 thousand seeds of selected legumes per ha. The mixed culture was established within one operation. DASA fertilizer (300 $kg \cdot ha^{-1}$; DASA® 26/13 Fertilizer CE, produced by Duslo corp., Slovakia; 18.5% w/w N-NH_4, 7.5% w/w N-NO_3, 13% w/w soluble S) was applied to all variants before the sowing, in a dose that was sufficient to cover the nutritional requirements of maize and did not limit the growth of legumes.

Plant biomass was sampled manually at a height of 18 cm above the ground. It was harvested at a BBCH 77–83 growth stage of maize (early milk to early wax ripeness) being collected from each repetition of the respective variants (Appendix A). Subsequently, a 15–20 mm cut was prepared using the Deutz-Fahr MH 6505 cutter (Deutz-Fahr, Lauingen, DEU). The cut was used to prepare model micro-silages in triplicate (Table 1). The preparation of micro-silages was the same for all variants. The cut biomass (8 kg) was placed in a micro-silage container (Ø150 mm × 1000 mm) with the inoculant (Silo Solve EF, CHr. Hansen, CZE; *Lactococcus lactis*, *L. plantarum*, *Enterococcus faecium*—dose 6.25 × 10^5 CFU per g of plant biomass) at a dosage of 5 g. This dose was dissolved in 3.5 L $H_2O \cdot t^{-1}$ and subsequently applied to the cut biomass. The prepared plant material was compacted using the pneumatic press with a force of 6000 $N \cdot m^{-2}$. Subsequently, the micro-silage was sealed and placed in an incubation room without access to light at a constant temperature of 28 °C (±1 °C) for 90 days. At the end of the incubation period, the micro-silages were opened and homogenized. Frozen samples of micro-silages were transported to the laboratory to perform their chemical analysis and fermentation tests.

Table 1 Prepared model silages

Variant	Content of maize (wt%)	Content of broad bean (wt%)	Content of white lupine (wt%)	Ratio of fresh matter of individual plants
M	100	0	0	1
B	0	100	0	1
L	0	0	100	1
MB	94.6	5.4	0	17.5:1
ML	76.9	0	23.1	3.33:1

M maize, *B* broad bean, *L* white lupine, *MB* maize + broad bean, *ML* maize + white lupine

Each silage made of mixed culture was made of 10 plants of maize and 10 plants of broad bean or white lupine

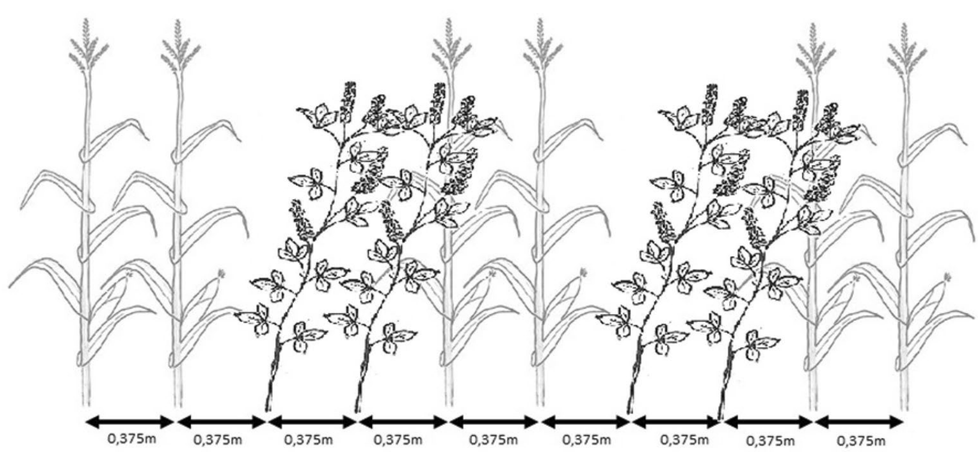


Fig. 1 Method of sowing a mixed culture of maize and selected legumes [35]

Production of digestate

Anaerobic fermentation (AD) of prepared silages was performed using fermentation batch tests in an automatic custom-made system. Each system included 5 L glass fermenters placed in a heated water bath with an adjustable constant temperature of 42 °C ± 0.1 °C. Each sample was fermented in triplicate. On the first day of the experiment, the fermenters were filled with 3 L of filtered (3 mm) inoculum obtained from the agricultural biogas plant processing maize silage and slurry, which is operated at low DM of 3–4%.

Two fermenters in each system served as a blank for determining biogas production from the endogenous inoculum. The initial organic loading rate was 5.5 g of volatile solids (VS) of introduced substrate per L. Retention time was 21 days. The biogas produced was measured daily using the liquid extrusion method (according to VDI 4630) with an acidified saturated NaCl solution as a barrier solution. The generated volume of biogas was converted to standard temperature and pressure (273.15 K and 1 bar). Each fermenter had a port to analyse the biogas composition. The Dräger X-am 5600 (Dräger, Germany) was used to analyse the biogas composition.

The produced digestate was subsequently analysed to determine the content of total carbon (TC) and total organic carbon (TOC), macronutrients (N, P, and K), dry matter (DM) and organic substances, crude fibre (CF), acid detergent fibre (ADF), neutral detergent fibre (NDF), and acid detergent lignin (ADL). TC and TOC were determined by dry combustion, using the TruSpec analyser (LECO, USA) according to ISO 10694: 1995 [36]. The determination of N, P, and K contents was performed in several steps. The individual samples underwent wet digestion according to [37]. The total nitrogen (TN) content was determined by the Kjeldahl method [38]. The P content was determined spectrophotometrically (Onda VIS V-10 Plus spectrophotometer, Giorgio Bormac, Italy) [39] and the K concentration was measured using the atomic absorption spectrometer Agilent 55B AA (Agilent, USA). The CF content was determined by the two-step hydrolysis with sulphuric acid and potassium hydroxide. The ADF extraction was performed using a solution of concentrated sulphuric acid and cetyltrimethylammonium bromide. Solution of sodium lauryl sulphuric and ethylenediamine tetra-acetic acid was used for the extraction of NDF. All analyses for CF, ADF, and NDF were performed on the ANKOM 200 Fibre Analyser (ANKOM Technology, USA). The ADL content was measured in accordance with [40]. The cellulose content was calculated as a difference between ADF and ADL [41], hemicellulose was determined as a difference between NDF and ADF [42].

Soil and pot experiment preparation

The scheme of the short-termed pot experiment is displayed in Fig. 2. Six variants were prepared in 4 repetitions. Following soil amendments were used for the laboratory-scale experiment: control and 5 different digestates. The digestates tested in the pot experiment were obtained from the anaerobic fermentation of 5 silages under laboratory conditions as described above. We used digestates which were prepared from five different silages: (1) maize, (2) broad bean, (3) white lupine, (4) maize + broad bean, and (5) maize + white lupine. The first three digestates were prepared by AD of monoculture silages. The last two digestates were produced by AD of silages formed by the biomass of two plants (maize + broad bean or white lupine) in different proportions (17.5:1, respectively 3.33:1). Their properties are described in detail in the chapter of Results—Quality of digestate.

Each of 36 experimental pots sized 1 dm^3 was filled up with 1 kg of soil substrate (topsoil from the rural area, sieved through a grid size of 2.0 mm and mixed with fine quartz sand—0.1–1.0 mm—1:1, w/w). Properties of the used topsoil, silty clay loam (Haplic Luvisol) collected from a locality near the town Troubsko, Czech Republic (49°10′28"N 16°29′32"E) are given in Table 2. The control soil variant was not amended, the other variants were supplied with digestates in amounts corresponding to 100 kg $N \cdot ha^{-1}$. Characteristics and dosages of tested digestates are presented in Fig. 2.

To achieve the same wetting of all pots at the beginning of the experiment, the control variant was watered with 50 ml of distilled water and digestate (Table 3) was filled with distilled water to make up 50 mL for the application to other variants.

The pot experiment with lettuce (*Lactuca sativa* L. var. *capitata* L.) cv. Smaragd was carried out under controlled conditions in the growth chamber Climacell Evo

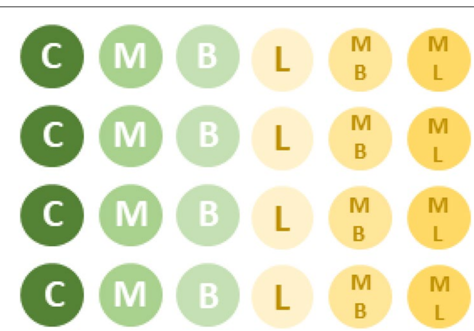


Fig. 2 Scheme of the pot experiment: C negative control, M maize, B broad bean, L white lupine, MB maize + broad bean (17.5:1), ML maize + white lupine (3.33:1)

Table 2 Properties of topsoil used for the pot substrate preparation

Parameter	Value	Unit	Parameter	Value	Unit
pH($CaCl_2$)	7.29		C/N	8.77	
TC	14.00	$g.kg^{-1}$	S	145	$mg.kg^{-1}$
TN	1.60	$g.kg^{-1}$	P	97	$mg.kg^{-1}$
$N_{mineral}$	65.72	$mg.kg^{-1}$	K	231	$mg.kg^{-1}$
N-NO_3	59.40	$mg.kg^{-1}$	Ca	3259	$mg.kg^{-1}$
N-NH_4	6.32	$mg.kg^{-1}$	Mg	236	$mg.kg^{-1}$

N-NO_3, N-NH_4 were determined according to ISO 15476: 2009; S, P, K, Ca, Mg were determined according to ISO 15178: 2000, ISO 14869–3: 2017, and ISO 13196: 2013

Other methods used are listed in Table 4.

Table 3 Characteristics and dosage of N in the tested digestates

Digestate	M	B	L	MB	ML
DM (%)	2.76	2.49	3.00	2.94	2.28
Dose for 100 kg N·ha^{-1} (t·ha^{-1})*	34.01	31.75	25.97	30.40	33.22
Dose per pot (g of fresh matter)	27.21	25.40	20.78	24.32	26.58

M maize, *B* broad bean, *L* white lupine, *MB* maize + broad bean (17.5:1), *ML* maize + white lupine (3.33:1)

*Only 70% of total nitrogen was converted to be accessible for pig slurry and digestate from the biogas plants for the purposes of the fertilization limit evaluation [43]

(BMT, Czech Republic) with blue-infrared (IR) illumination (optimized for photosynthesis intensification)—light intensity 20 000 lx [44]. Five lettuce seeds were sown at about 2 mm depth in each pot and incubated in the growth chamber under defined conditions: temperature 18/22 °C (night/day), a 12 h photoperiod, relative air humidity 70% [45]. The pots were placed in the growth chamber randomly. After 14 days, the seedlings were unified and a representative one was left in each pot. All plants were manually watered with 50 mL of distilled water every other day. Once per week, the pots were rotated variably [46]. The individual seedlings were harvested 6 weeks after sowing. At the end of the pot experiment, the soil samples were collected, and the plant and soil parameters were determined (Table 4).

Determination of plant and soil parameters

Plant and soil samples were analysed to assess selected parameters which are summarized in Table 4.

Statistical analysis

Data processing and statistical analysis were carried out with the help of the statistical program R version 3.6.3. [50] together with additional packages “ggplot2” [51] for creating all statistical graphs.

Multivariate analysis of variance (MANOVA) and principal component analysis (PCA), with the dependence of different treatments, were used for modelling the relation between the soil properties and the selected treatments, with the help of additional packages “factoextra” [52] and “FactoMineR” [53]. One-way analysis of variance (ANOVA) and Duncan’s multiple range test from the “agricolae” package [54] at a significance level of 0.05 were used to detect the difference among the treatments. Factor level means were calculated (with 95% confidence interval–CI) using the “treatment contrast”. Partial eta-squared (ηp2) from the “BaylorEdPsych” package [55] was used to measure the effect size, and the Pearson correlation coefficient (with 95% CI) was applied to determine the linear dependence among the soil properties.

Results and discussion

Quality of digestate

Parameters indicating the quality of digestate which was prepared by the same fermentation process (identical type of fermenter and inoculum) for each variant but with a different input of biomass, are summarized in Tables 5, 6 and 7.

Table 4 Determination of plant and soil properties in the pot experiment

Parameter	Method–reference	References	Abbreviation
Aboveground plant and root biomass dry	gravimetrical (weight of lettuce shoot and roots dried at 60 °C), analytic scale	[47]	AGB, root dry
Basal soil respiration	MicroResp® device, official instructions (Technical Manual v2.1, The James Hutton Institute)	[48]	BR
Substrate induced soil respiration	MicroResp® + inducers: D-glucose (Glc), N-acetyl-β-D-glucosamine (NAG), D-trehalose (Tre), L-alanine (Ala), L-lysine (Lys), L-arginine (Arg)	[48]	SIR (Glc-, NAG-, Tre-, Ala-, Lys-, Arg-)
Total soil carbon	Dry combustion (ISO_10694 1995) using LECO TruSpec analyser (MI USA)	[36]	TC
Total soil nitrogen	Dry combustion (ISO_13878 1998) using LECO TruSpec analyser (MI USA)	[49]	TN
Total soil C/N ratio	Calculation from the TC and TN values		C/N

Table 5 Chemical composition of used digestates – content of nutrients

Digestate	DM [%] ± SE	TN [$g \cdot kg_{TS}^{-1}$] ± SE	K [$g \cdot kg_{TS}^{-1}$] ± SE	P [$g \cdot kg_{TS}^{-1}$] ± SE
M	$2.76^{ab} \pm 0.06$	$152.17^{b} \pm 7.54$	$6.52^{b} \pm 0.21$	$22.10^{b} \pm 0.56$
B	$2.49^{b} \pm 0.06$	$180.72^{a} \pm 8.36$	$8.83^{ab} \pm 0.40$	$23.29^{b} \pm 0.61$
L	$3.00^{a} \pm 0.11$	$183.33^{a} \pm 6.94$	$9.67^{a} \pm 0.19$	$32.00^{a} \pm 0.84$
MB	$2.94^{a} \pm 0.08$	$159.86^{b} \pm 5.89$	$7.82^{b} \pm 0.34$	$31.63^{a} \pm 0.86$
ML	$2.80^{ab} \pm 0.05$	$153.57^{b} \pm 4.12$	$10.00^{a} \pm 0.54$	$22.86^{b} \pm 0.72$

M maize, *B* broad bean, *L* white lupine, *MB* maize + broad bean (17.5:1), *ML* maize + white lupine (3.33:1), *DM* dry matter, *TN* total nitrogen content

Average values of available nutrients and dry matter are displayed ($n = 3; \pm SE$)

The values of nutrient contents were converted to the value of dry matter

Different letters indicate statistically significant differences ($p \leq 0.05$) in the ANOVA posthoc Duncan's multiple range test between individual variants in the content of selected parameter

Table 6 Chemical composition of used digestates—contents of organic compounds CF, ADF, NDF, ADL

Digestate	CF ($\%_{TS} \pm SE$)	ADF ($\%_{TS} \pm SE$)	NDF ($\%_{TS} \pm SE$)	ADL ($\%_{TS} \pm SE$)
M	$10.87^{a} \pm 0.42$	$15.58^{b} \pm 0.42$	$22.82^{b} \pm 0.91$	$7.49^{c} \pm 0.32$
B	$9.24^{b} \pm 0.40$	$16.06^{b} \pm 0.46$	$29.32^{a} \pm 0.93$	$10.31^{b} \pm 0.48$
L	$5.67^{c} \pm 0.19$	$24.00^{a} \pm 0.77$	$30.67^{a} \pm 0.84$	$14.33^{a} \pm 0.38$
MB	$6.80^{c} \pm 0.34$	$23.47^{a} \pm 0.90$	$28.57^{a} \pm 1.04$	$10.09^{b} \pm 0.30$
ML	$7.14^{c} \pm 0.20$	$11.43^{c} \pm 0.41$	$18.21^{c} \pm 0.71$	$7.14^{c} \pm 0.20$

M maize, *B* broad bean, *L* white lupine, *MB* maize + broad bean (17.5:1), *ML* maize + white lupine (3.33:1)

Average values ($n = 3; \pm SE$) of the contents of organic substances CF (crude fibre), ADF (acid detergent fibre), NDF (neutral detergent fibre), ADL (acid detergent lignin) are displayed in the individual types of digestate

Values of the contents of organic substances are converted to dry matter

Different letters indicate statistically significant differences ($p \leq 0.05$) in the ANOVA posthoc Duncan's multiple range test between individual variants in the content of selected parameter

Table 7 Chemical composition of used digestates—contents of organic compounds

Dig	Cellulose [$\%_{TS} \pm SE$]	Hemicellulose [$\%_{TS} \pm SE$]	TC [$\%_{TS} \pm SE$]	TOC [$\%_{TS} \pm SE$]	C/N ± SE
M	$8.09^{bc} \pm 0.43$	$7.24^{b} \pm 0.56$	$29.33^{b} \pm 0.01$	$29.25^{b} \pm 0.08$	$1.93^{a} \pm 0.09$
B	$5.76^{cd} \pm 0.58$	$13.25^{a} \pm 0.46$	$28.91^{c} \pm 0.05$	$28.80^{c} \pm 0.04$	$1.61^{bc} \pm 0.08$
L	$9.67^{b} \pm 0.67$	$6.67^{c} \pm 0.33$	$28.85^{c} \pm 0.04$	$28.74^{c} \pm 0.04$	$1.58^{c} \pm 0.06$
MB	$13.38^{a} \pm 0.69$	$5.10^{c} \pm 0.20$	$29.64^{a} \pm 0.03$	$29.54^{a} \pm 0.03$	$1.85^{ab} \pm 0.07$
ML	$4.28^{d} \pm 0.55$	$6.79^{c} \pm 0.41$	$29.58^{a} \pm 0.01$	$29.45^{a} \pm 0.01$	$1.92^{ab} \pm 0.05$

M maize, *B* broad bean, *L* white lupine, *MB* maize + broad bean (17.5:1), *ML* maize + white lupine (3.75:1)

Average values ($n = 3; \pm SE$) of cellulose and hemicellulose in the individual types of digestate are displayed

The content of organic substances is converted to dry matter

Different letters indicate statistically significant differences ($p \leq 0.05$) in the ANOVA posthoc Duncan's multiple range test between individual variants in the content of selected parameter

The highest DM was recorded in the variants maize + broad bean and lupine (+7% and + 9% compared to maize, respectively). These values were significant in relation to the lowest DM content which was detected in broad bean (-10% compared to maize). The DM content then decreased in the following order maize + lupine > maize > broad bean. However, there were no significant differences between these variants. Overall (Appendix B: Table 8), the values of DM ranged from 2.37 to 3.17% (min ↔ max).

Compared to the DM content, the P content in the individual digestates was more variable (Appendix B: Tables 8 and). The difference between the minimum and maximum values was greater than 50%, which was more than in the case of DM (33% difference between min and max). Demonstrably, the highest values (> 30 g $P \cdot kg_{TS}^{-1}$)

were found in lupine and maize + broad bean (+ 45% and + 43% compared to maize, respectively), which was conclusive against the remaining variants. In the remaining variants (maize, broad bean and maize + lupine), the P content values were around 23 $g \cdot kg_{TS}{}^{-1}$. The lowest P content was found in the maize variant, but this was not conclusive against the variants of broad bean and maize + lupine. Regarding the K content, significant differences were found between the individual variants. The highest K content was measured in the maize + lupine variant. The difference was significant when compared to the variants of maize and maize + broad bean. This value was by + 53% (on average) higher compared to the maize variant, where the demonstrably lowest K content was found.

The highest TN content (Table 5) was recorded in the lupine and broad bean variants, increased by 20% and 19% in comparison to maize, respectively. The lowest content was detected in the maize variant similarly as at K and P nutrients. This was evident in broad bean and lupine. Although the maize + lupine and maize + broad bean variants showed higher values of TN content (+ 1.5%) compared to the maize variant, not all differences were statistically significant.

The displayed values of chemical contents of the applied digestates – nutrient contents (Appendix B) conclusively confirm that different input biomass intended for fermentation affected the content of fermentation residue. It was reported previously that the quality of feedstock affects not only the production of biogas but also the chemical composition of fermentation residue [56, 57]. The detected values confirm that the addition of legumes positively affected increase in the contents of P, K and N compared with variants, where digestate was made of only maize, although the differences were not always statistically significant. These results demonstrate the potential positive effect of legumes used for biogas production on the quality of the resulting digestate. Different plant species used to produce silage affected its quality. This is confirmed in the experiment by Kintl et al. [35], who focused on the effect of legume addition into maize silage on biogas production and silage quality. The authors noticed that the biomass of legumes increased the content of N substances in silage. We evidenced that broad bean and white lupine significantly enriched the digestate with N (by 19% and 20%, respectively) after mono-fermentation, as well as less demonstrably but apparently in the mixed culture digestate (maize + broad bean). Similarly, a higher content of crude and soluble nitrous substances in the legume feedstuff silages was reported as compared to the feed maize silage [58]. On the other hand, the content of fibre and other anti-nutrition substances increased in the mixed culture digestate (maize + broad bean) and white lupine digestate. It partly corresponds to the development of DM content (Table 5), where the highest amount was detected in the mixture of maize + broad bean and lupine digestate. In the agronomic model, where legumes are used to prepare silage, the use of legumes has a positive effect on increasing the efficiency of N use. This increases the N content in silages and subsequently in the digestate, which was not obtained from mineral fertilizers, but as a product of natural biological N fixation [59]. The research of the influence of various types of plant biomass from sustainable production (especially legumes) represents a new area with the insufficient amount of knowledge. However, from previous studies and experiments [60–62], it is obvious that the use of legumes as a source of biomass for biogas plants leads to the increased quality of digestate, especially the N, P and K contents, a pattern similar to the one obtained in the presented study (Appendix B: Table 9).

In addition to nutrient contents (Table 5), organic substances were observed (Table 6), represented by selected indicators (CF, ADF, NDF, ADL) corresponding to available and relatively easily degradable saccharides (e.g., hemicellulose), even more complex substances (e.g., lignin) which are resistant to microbial decomposition [63]. CF indicates the content of structural carbohydrates, which are an important source of energy for microorganisms in various processes (anaerobic fermentation, fermentation of plant biomass in the digestive tract of ruminants, etc.), but can have an inhibitory effect on microbial activity due to the content of less degradable substances [63]. The significantly highest value of CF content was in the maize variant ($10.87\%_{TS}$), compared to all other variants. The second highest CF content was found in the broad bean variant ($9.24\%_{TS}$), and this value was significantly different from all other variants. Other variants (white lupine, maize + broad bean and maize + white lupine) showed similar CF contents with no significant differences (Table 6) These differences in the crude fibre contents between the single-fermented maize digestate and both mixed culture digestates and the broad bean digestate corresponded to the presumption that legume biomass contains a lower fibre content than maize as well as higher amounts of protein [64]. However, the relation between the crude fibre content in the broad bean and lupine digestate was contradictory to the referred CF content in the non-fermented biomass, which was found lower in broad bean than in white lupine [65].

The CF parameter was also supplemented with the content of ADF and NDF. The ADF value was the highest in the white lupine and maize + broad bean variants, increased by 54% and 51% compared to maize, respectively. Again, there was a significant difference compared to the remaining variants. The significantly lowest ADF content was measured in the maize + white lupine variant (-27% as compared to maize). The NDF content in the digestates ranged from 16.79%$_{TS}$ (minimum) to 32.33%$_{TS}$ (maximum, Appendix C: Table 10). The highest average values (> 28.5%$_{TS}$) were found in the variants of white lupine, broad bean and maize + broad bean, these values were significantly higher than in the variants of maize (22.82%$_{TS}$) and maize + white lupine (18.21%$_{TS}$). The ADF and NDF values were previously found higher in the white lupine plant biomass than in the broad bean biomass [65]. However, the contradictory result of increased ADF content in the mixed maize + broad bean digestate, which was higher compared to both respective monocrop digestates (maize and broad bean), was novel. We found this observation related to the significantly highest content of cellulose in the maize + broad bean digestate, which both findings may indicate a lower rate of the degradation of complex C compounds in the respective fermentation process. The content of ADL showed the greatest variability, both in terms of total values (Appendix C: Table 10) and in terms of individual variants (Appendix C: Table 11). ADL values decreased in the following order: white lupine > broad bean > maize + broad bean > maize > maize + white lupine. The white lupine variant reached the demonstrably highest ADL content compared to the other variants. The higher content of insoluble polymers (similar to ADL) was referred to be significantly higher in the white lupine biomass than in the broad bean biomass [66]. Significant differences were found between the remaining variants of broad bean and maize + broad bean vs. maize and maize + white lupine. The differences among the respective digestates follow out from the character of used silages which were prepared from different crops or from their mixtures. Each crop belonged to a different family. Maize belongs to the family of *Poaceae*, similarly as the other cereals (e.g., wheat). *Poaceae* have a higher content of starch and sugar and a lower content of fibre and proteins in the grain. In contrast, legumes which belong in the family of *Fabaceae* contain high amounts of N-substances in the form of proteins in the grain [66, 67]. Moreover, legumes also contain a certain amount of antinutritional substances that are difficult to be degraded by microbial activity; one of the most important ones is coumarin [29]. The content of antinutritional substances differs in the individual *Fabaceae* species, and plants used in the experiment feature lower coumarin concentrations [27, 29]. All these substances affect the qualitative parameters of silage [27–29] and it can, therefore, be presumed that they showed also during the process of fermentation and hence in the composition of digestate fermentation residue.

Significant differences between the variants were noticed regarding the content of organic substances (cellulose, hemicellulose), TOC and TC in the respective digestates (Table 7). The largest differences (highest average cellulose, TC, TOC and lowest hemicellulose) were found in the digestate of maize + broad bean compared to the other variants. The results of hemicellulose showed a much lower variability among lupine, maize + broad bean, and maize + lupine. The highest content (13.25%$_{TS}$, + 83% as compared to maize) was found in the broad bean variant which is in contrast to the low content of cellulose in this variant (5.76%$_{TS}$, -29% as compared to maize). Other variants showed minimal differences and the hemicellulose content was at the same level in the white lupine, maize + broad bean variants, and maize + white lupine. Only a slight increase was found in the maize variant. TC values in the individual digestates ranged from 28.85% to 29.64%. TOC values varied within a similar range. Despite the relatively narrow range of the measured values, significant differences were detected due to the low variability of TC and TOC contents in the individual variants. The highest content of TC and TOC was demonstrably measured in the mixed culture digestates: maize + white lupine TC 29.58%$_{TS}$, TOC 29.45%$_{TS}$ and maize + broad bean TC 29.64%$_{TS}$, TOC 29.54%$_{TS}$. In contrast, silages of legume monocultures showed the significantly lowest values of TC and TOC content as compared to the maize + white lupine and maize + broad bean variants.

Furthermore, the ratio of TC and TN was monitored in the individual digestates (Table 7). There was no significant difference in the C/N between the conventional digestate from the sole maize biomass and the digestate from the mixed culture. This is crucial, because the C/N ratio is a significant indicator of digestate quality [68, 69]. According to some studies [18, 35, 59], the addition of silage prepared from legumes may lead to changes in digestate properties, i.e., to the reduced C/N ratio, because the biomass of legumes contains a larger amount of N substances than maize. This was not confirmed in our experiment, which data suggest that the organic N in legumes was partially lost during anaerobic fermentation. It was likely converted into ammonia which has a strong inhibitory effect on microbiological conversion via proton imbalance or interference with the metabolic enzymes of microorganisms [70]. However, the conclusions of our study confirm significantly lower C/N ratio values in digestates made of legume biomass (variants broad bean and lupine) compared to digestate from the sole maize silage. Another confirmation of the positive effect on the N content in the digestate using silage prepared from the biomass of legumes is the data displaying TN in the digestate (Table 7), where values of the variants with legumes were significantly the highest. It is obvious that the C/N ratio values are lower than standard values measured in conventional biogas plants. These mostly reach values 5–9 depending on the fermented material [69]. In our experiment, digestate used as an inoculum was filtered to remove particles larger than 3 mm and the test raw material was added in a low amount to avoid one-time overloading of the fermenters. Therefore, the resulting digestate can show slightly different values in the C/N indicators compared to operational/continuous biogas plants.

The content of different forms of fibre (ADF, NDF) and lignin (ADL) varied in the digestate depending on the type of input silage. The *Zea mays* L. plant has been bred into various silage hybrids, which have top properties supporting its high biogas production [69]. It was confirmed also in the experiment by Kintl et al. [35], where the silage of maize monoculture showed lower contents of CF, ADF, ADL and a higher NDF content compared to silage made of legume monoculture. This suggests that using legumes can lead to the enhancement of CF, ADF and ADL contents in the fermenter and subsequently in the digestate. When a mixed culture silage is used, the change can be observed (7). In our experiment, the maize variant showed lower ADF and ADL contents as compared to the broad bean, white lupine, and maize + broad bean variants, i.e., substances indicating the content of organic compounds were more resistant to decomposition during anaerobic fermentation [71, 71], than variants consisting of legumes only. However, fermentation of biomass from the mixed culture with white lupine resulted in a decrease of these substances (Table 6). Contrarily, using mixed culture with broad bean increased NDF in the AD process compared to maize culture. The presence of NDF has a potential impact on the digestate quality, because its higher content indicates complex (cellulose, lignin) and less complex saccharides [72] convenient to stimulate soil microorganisms [73]. The content of cellulose and hemicellulose could not be related to the effect of monoculture or mixed culture used to produce digestate. Cellulose is a polysaccharide made of only glucose units, while hemicellulose is formed by glucose, pentose and also alduronic acid [72]. While the content of hemicellulose and cellulose was variable and did not indicate a direct effect of the use of mixed silage, the content of TC and TOC was affected. Moreover, the TOC values of the individual digestates corresponded to the TC values. After the anaerobic fermentation, the digestate consists of d less degradable organic matter as most of the labile organic substances were decomposed during the anaerobic fermentation [5]. At present, there is insufficient knowledge about the influence of legumes on the process of anaerobic fermentation and the quality of digestate. There are mainly studies aiming at a potential use of legumes in anaerobic fermentation to reduce energy consumption and greenhouse gas production [74] or the profitability of their use for biogas [59]. On the other hand, there are studies [56] confirming that quality, thus the chemical composition, is derived from the properties of biomass used in the process of anaerobic fermentation. It can be assumed that the addition of leguminous biomass into silage intended for the fermentation process can improve digestate quality, i.e., increase the content of TC and TOC and subsequently the C/N ratio which represents the main problem for long term usability of N containing substances in digestate by crop [5]. Thus, it is necessary to study the effect of legume silage addition on digestate quality, because the results indicate a potential effect on an increase in N substances and thus decrease in C/N which is not desirable.

Soil respiration

Basal soil respiration (BR) (Appendix E) ranged from 0.16 to 0.28 μg $CO_2 \cdot g^{-1} \cdot h^{-1}$. BR of all digestate-amended variants was significantly lower in comparison to the control variant (0.28 μg $CO_2 \cdot g^{-1} \cdot h^{-1}$), where the highest value was found. Among all tested digestates, the most enhanced BR was achieved with the mixed culture of maize + broad bean. Its value was significantly higher than in the maize + white lupine and maize variants. The significantly lowest value compared to all other variants was found in the variant, where digestate was made of maize + white lupine.

BR values can be considered as directly proportional to the metabolic activity of soil microorganisms [76]. The measured BR values in the experiment indicate that the application of digestate generally did not contribute to the increase of microbial activity in the soil, as it was always lower than in the control variant. This finding is of interest, because there are studies [77] confirming a positive effect of digestate application on the microbial activity in the soil. However, other studies [77–79] point out that soil properties can affect the structure, variability and activity of microorganisms more than only digestate or other fertilizer application [7879] finding similar values as in our experiment, i.e., a higher level of BR in the untreated variant compared to the variant with the digestate application. According to Gómez-Brandón et al. [79] and Odlare et al. [80], the reason is readily metabolized C in the digestate. It is easily and quickly used by microorganisms, and causes increased BR within a short time. However, in the long run, the effect on the microbial activity is negligible. In our case, BR was measured at the end of the experiment, not continuously. Therefore, lower BR values were found in the variants with the digestate application. Microorganisms probably depleted metabolizable C soon after the application of digestate and had no other energy source at the end of the experiment. This can be indicated by the composition of digestate in terms of organic matter content (Table 7), where the content of less degradable substances (expressed by ADF or ADL) prevailed.

On the other hand, there were significant differences between the individual types of digestate. These were probably due to the different composition of individual digestates (Table 5). In the individual digestates, I highest BR values were reached by those that showed an increased content of DM and ADF: maize + broad bean and white lupine. Thus, there is a presumption that these digestates contained higher amounts of stable C than the other ones, which persisted in the soil longer after the digestate application. Thus, it could have been metabolized by soil microorganisms for a longer period [81] leading to increased BR values at the end of the experiment.

In addition to BR, SIR values were monitored, too (Fig. 3B-G). The development of the values shows that there is a similar trend in SIRs as in BR. Thus, after the addition of most substrates (trehalose, Fig. 3C), glucosamine (Fig. 3D), and lysine (Fig. 3F), the control variant showed the highest SIR value, or reached similar SIR values (glucose, Fig. 3B), alanine (Fig. 3E), arginine (Fig. 3E) as the variants with the digestate application. This condition can be also explained according to [79, 80] by the fact that the application of digestate supports microorganisms only in the short term. Similar conclusions were reported by Abubaker et al. [81] in the measurement of soil respiration as a significant increase of microbial activity was observed after the application of digestate, which was, however, attenuated after a certain time.

Regarding partial differences between the individual forms of digestate, the maize + white lupine variant showed the lowest values after the addition of all substrates again. Furthermore, SIRs (excluding Arg-SIR) were as low as in the BR in the maize variant. The other variants were at the same SIR level, while the broad bean and maize + broad bean variants showed the highest SIRs values (except Arg-SIR) among the digestates again. According to Bloem et al. [76], SIR represents the potential microbial activity of soil organisms. Based on the measured values, it is possible to assume that the

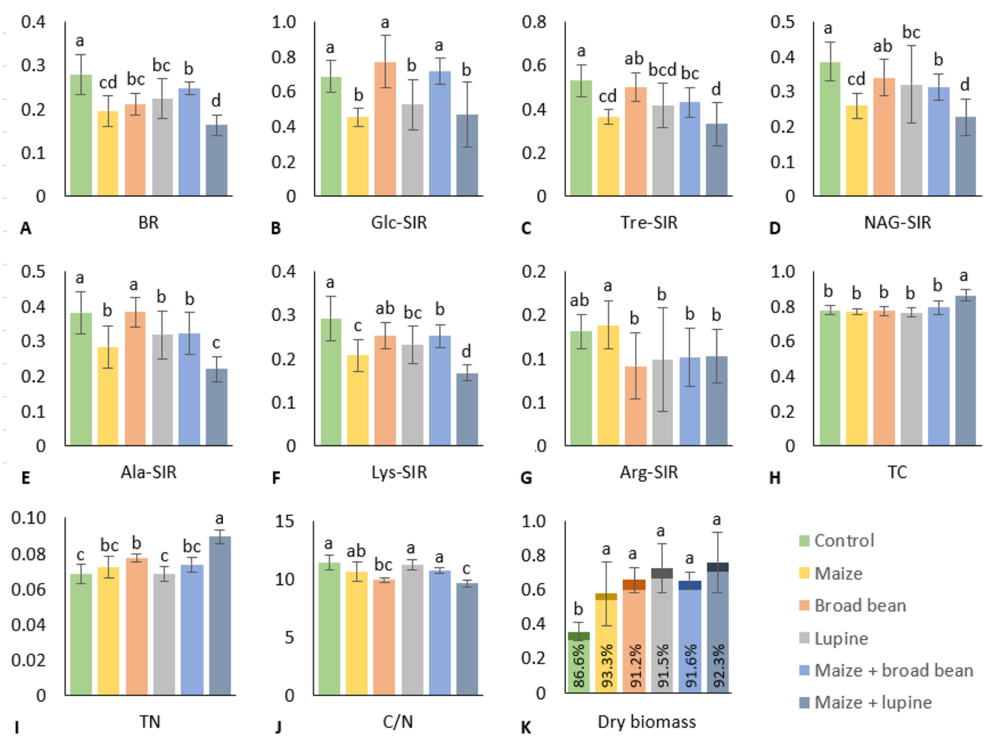


Fig. 3 Soil properties and plant biomass in the pot experiment. Legend: **A** basal respiration [μg CO_2 g^{-1} h^{-1}], **B**–**G** substrate induced respiration [μg CO_2 g^{-1} h^{-1}], **H**–**I** total element [%], J) C/N ratio [−], **K** dry biomass [g·plant^{-1}] – light colour Aboveground biomass (AGB) [% of total weight] and dark colour Root. Different letters indicate statistically significant differences ($p \leq 0.05$) in the ANOVA post-hoc Duncan's multiple range test between individual variants in the content of selected parameter

application of digestate prepared by the fermentation of enriched maize silage (maize + broad bean), or only from broad bean, can positively affect the microbial activity in the soil through the increased content of organic matter (Table 7). It is, therefore, clear that the type and quality of the input biomass (silage) change the effect of digestate on the microbial activity in the soil.

The similar values of SIR were recorded by Gómez-Brandón et al. [79] who tested SIR after the addition of glucose. Compared to BR, they observed increased respiration in the variant treated with digestate at the same level as in the control variant. In our experiment, a similar trend was observed but only at a part of the digestate. This can be related to digestate composition.

Soil carbon, nitrogen, and C/N

The measured values of average TC (Fig. 3H) in soil samples from the individual variants ranged from 0.78 wt% to 0.86 wt%. These values can be considered as balanced, because only one significant difference was found. The maize + lupine variant showed the significantly highest TC compared to all other variants. Moreover, the variance of the TC content in the soil was low (Appendix E, F, G).

The TN content showed a greater variability across the variants than the TC content although the variance of values within the individual variants was lower there (Appendix E, Appendix G). The lowest average values (< 0.07 wt%) were found in the control variant and in the white lupine variant compared to the broad bean and maize + lupine variants. On the other hand, the significantly highest TN content was found in the variant of maize + white lupine (0.09 wt%).

The level of TC in the topsoil (0–20 cm) is a key parameter for soil quality, fertility and the nutrient pool [82]; thus, the soil C balance is crucial for greenhouse gas emissions in the environment as well as for the plant nutrition. Likewise, the content of TN in the soil is an important indicator of the condition of environment [82, 84]. This is because the content of C and N substances in agricultural soil and their composition (quality) is directly proportional to the way how the land is used [83, 84, 86] and is decisive for the availability of nutrients to plants [85, 87]. The measured TC values do not indicate that the addition of legumes to maize silage influences the quality of digestate after the fermentation process, which would be reflected in changes in the soil composition after their application. This finding corresponds with the study by Jensen et al. [62] who point to a smaller effect of the addition of individual plant species (e.g., the family of *Poaceae* and *Fabaceae*) on the C content in digestate. At the same time, the studies raise a possible effect of the addition of legumes on increasing the N content in the input silage and thus subsequently in the produced digestate. Variants with the addition of legume to the fermented silage showed the same or a higher TN content due to the digestate application as compared to the control soil or digestate prepared by fermentation of maize silage only (Fig. 3I). Furthermore, these digestates increased the soil TN values, because they contained more N-substances when total N (Table 5) during the application is considered. Although the digestate dose was converted to the N content, the forms of N in the respective digestate types were likely determined in relation to the type of input feedstock, similarly as described by Herrmann et al. [88]. It follows from the above that the composition of silage entering the fermentation process affected the N content in the digestate. Its highest amounts were observed in digestate variants, where the silages were prepared from legumes. Nevertheless, the highest TN in the soil was found in the maize + lupine variant. Therefore, the lowest C/N ratio was found (expectedly) in this variant. The lower the C:N ratio is, the more rapidly nitrogen is released into the soil for immediate crop use, which results in the higher plant biomass, as it was revealed for the dry biomass value of maize + lupine variant.

Furthermore, the soil C/N ratio was calculated for the individual variants (Fig. 3J). The highest values (> 10:1) were found in the control variant, white lupine, and maize + broad bean variants. The other variants had a C/N ratio below 10:1. The lowest value (demonstrably compared to all other variants except broad bean) was found in the maize + white lupine variant (9.65:1). The C/N ratio in the soil indicates the process of soil organic matter (SOM) decomposition [89]. When the value falls below 10, the decomposition accelerates. The optimal value for arable land is 10–13:1 and should not exceed the limit of 20:1 [90]. The optimal representation of C and N substances can be maintained mainly by the application of organic fertilizers and sustainable farming [62, 90].

Digestates are known for their low ratio of C/N. The process of anaerobic fermentation is related to C loss. Thus, a great increase in soil TC after digestate application cannot be expected. Contrarily, it positively affects the content of TN, which was confirmed by our experiment, and leads to lowering ratio of C/N in the soil by C decrease which was decomposed by microorganisms within the fermentation process [69, 91]. It also explains a slight C/N decrease in the individual variants fertilized by digestate in comparison with the control variant. Furthermore, the effect of different digestate addition to the soil was not clearly determined in terms of C content and subsequently C/N. The addition of legumes into silage (input biomass) mainly affects the content of N-substances or fibre [35], basic nutrients [62], and thus subsequently the composition of digestate (Tables 5 and

6). This was reflected in the higher N_{min} content in the digestates and probably also in the soil used in the pot experiment. Due to the small differences in C/N among the individual digestates, the effect of the addition of legumes could not be determined.

Plant biomass production

At the end of the pot experiment, the total biomass production of the indicator plant was determined (Fig. 3K). Significantly higher values of biomass yield (>0.57 g) were found in the variants fertilized with digestate than in the control variant. However, no significant difference was found among the individual digestates, even though the average values ranged from 0.57 (maize variant) to 0.76 g (maize + lupine variant). The absence of significant differences was due to the increased variance of measured values (Appendix G). All variants were fertilized with the same dose of N (Table 2), but with a different dosage of other nutrients (Appendix D: Tables 12 and 13). The data did not confirm the expected significant difference in yield. Digestates with the highest contribution of nutrients to the soil, i.e., white lupine (+25 kg $P{\cdot}ha^{-1}$ and +8 kg $K{\cdot}ha^{-1}$), maize + white lupine (+28 kg $P{\cdot}ha^{-1}$ and +9 kg $K{\cdot}ha^{-1}$) and maize + broad bean (+28 kg $P{\cdot}ha^{-1}$ and +7 kg $K{\cdot}ha^{-1}$) showed higher yield values, but the differences were not statistically significant.

The positive effect of digestate application on plant growth is well known and described in Al Seadi et al. [56], Coelho et al. [57] and is very often compared to that of conventional mineral N fertilizers [92]. Although the results confirm (Table 5) that the addition of legumes to silage used for biogas production led to the increased content of N and other nutrients in the digestate, a positive effect on the plant biomass production could not be proven (at a level of $P<0.05$). Above all, there are obvious differences between the variant with the application of digestate only from the maize silage and silage prepared from the mixed culture (+14% in maize + broad bean and +33% in maize + lupine), these differences are not significant. In any case, the potential for the addition of leguminous species to silage is obvious (given the contribution of nutrients to arable land) but must be further tested. The possibility of using legumes to increase the positive environmental impact of anaerobic fermentation was confirmed by Jensen et al. [62], Stinner et al. [75], but the study of partial aspects has not been realized yet.

Relation between individual indicators of soil microbial activity and soil fertility

Evaluation of interrelationships with comparable soil parameters in individual experimental variants of the pot experiment is expressed by the PCA graph in Fig. 4.

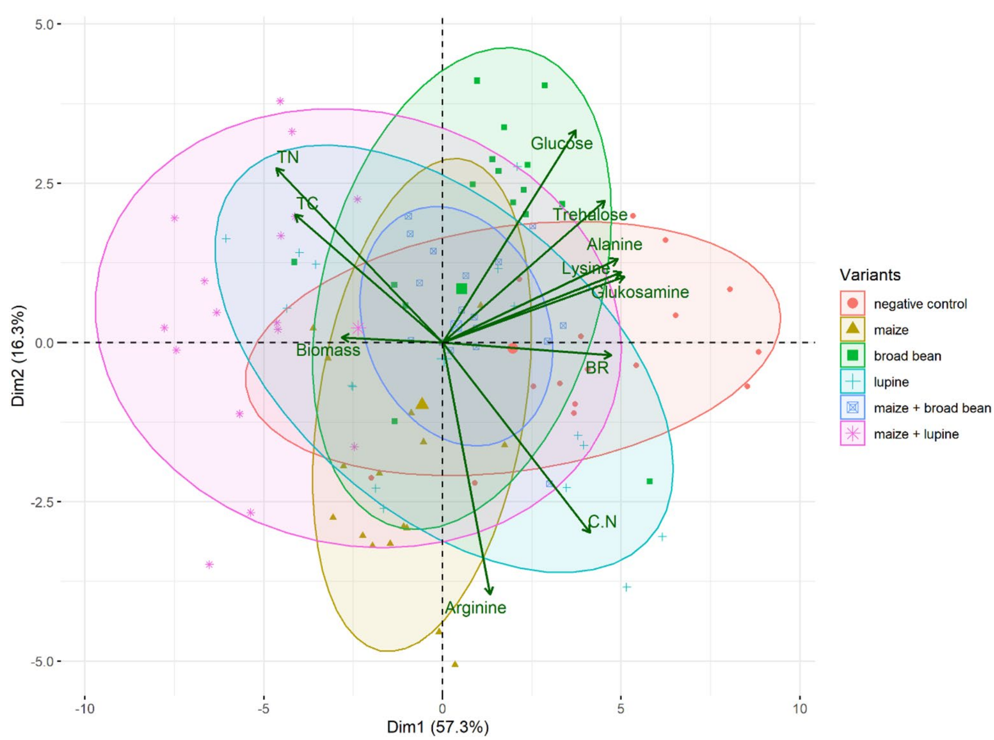


Fig. 4 PCA plot of relations between the soil properties of all variants

The numerical expression of the interdependence of soil properties is shown in Fig. 5 on the Pearson's correlation coefficient values.

The results of the statistical analysis show that two main factors were identified (Fig. 4). These describe more than 75% of the variability of measured values. Factor 1 (61.3%) correlated positively (>0.5) with SIR (alanine, trehalose, lysine, glucosamine) and, conversely, showed a weak negative correlation with TC and TN. Based on these data, it can be assumed that this factor explains the variability caused by the type of used digestate, because, e.g., the SIR value (Fig. 3B-G) was evidently affected by the application of different digestate types. Factor 2 (15.4%) exhibited only very weak to negligible correlations. They were positive to TN, TC and SIR (glucose and trehalose) parameters. Thus, it can be assumed that this factor explains the variability caused by the type of soil used or conditions under which the experiment was performed (temperature, lighting, humidity, etc.).

Correlation matrices are made to describe the identification of the relationship between individual parameters in more detail (Fig. 5). It is evident that there was a strong positive dependence between the individual types of SIR (R>0.6). SIR determination consists in measuring the microbial respiration of soil samples after their treatment with an excess of easily degradable energy and nutritional source, which serves to induce microbial activity [93]. Thus, SIR is considered to be a parameter expressing the potential microbial activity of bacterial and fungal biomass [94]. Therefore, it is no surprise that all SIR variants correlated positively with each other, as they expressed the reaction of not very variable soil microflora to the addition of easily degradable energy substrates. The strongest correlation was found between the

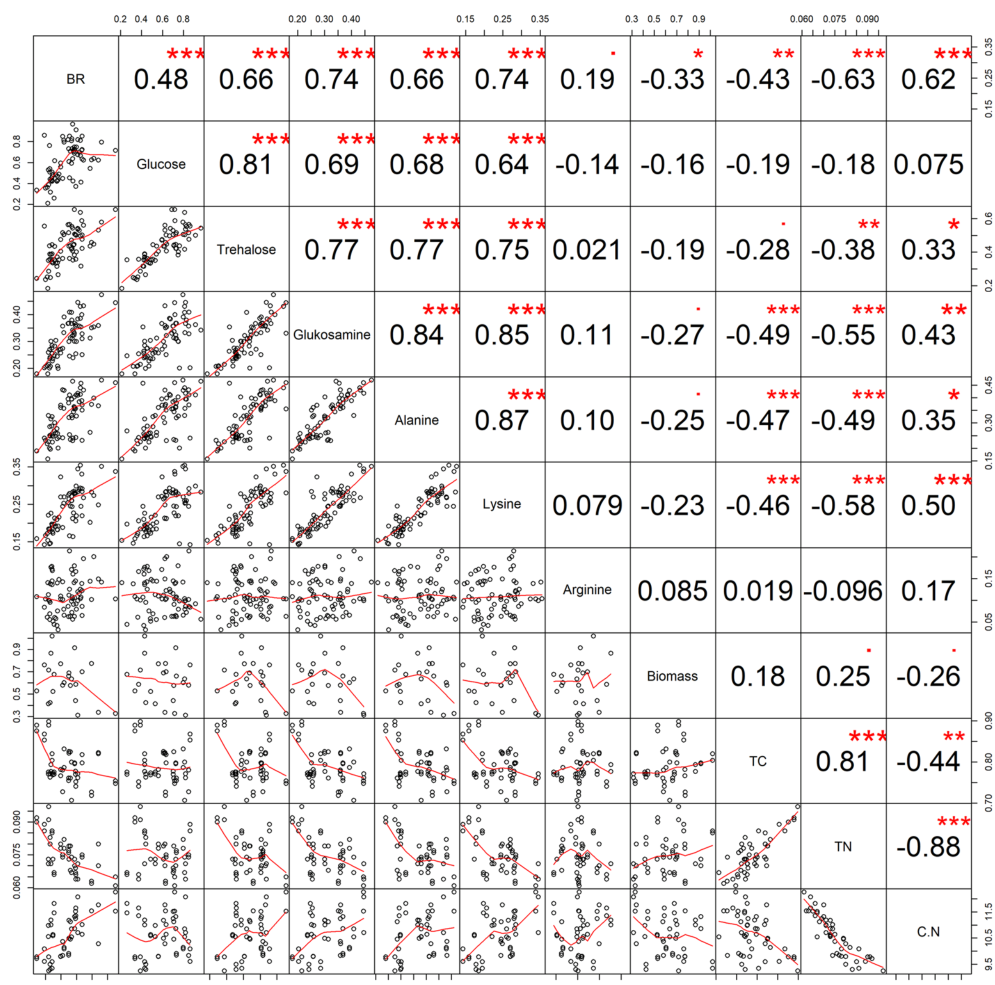


Fig. 5 Pearson's correlation matrix of soil properties. Legend: statistically significant difference at a significance level of 10%, * statistically significant difference at a significance level of 5% ** statistically significant difference at a significance level of 1%, *** statistically significant difference at a significance level of 0.01%

following pairs: glucose and trehalose, glucosamine and alanine, glucosamine and lysine, and alanine and lysine. These correlations were caused by similarities in the composition of individual substances and that is why the same positive effect on SIR could be observed. Furthermore, a positive correlation was found between BR and SIR, but the dependence was demonstrably lower there. This can be explained by the effect of soil water content and available nutrients on BR [92, 94]. At the end of the experiment, when BR and SIR were measured in the soil samples, nutrients had been already depleted. Thus, the measured BR was lower and SIR (after the addition of easily degradable nutrients) was higher. The difference in values pointed to the reducing strength of the relationship (R from 0.48 to 0.74).

Interestingly, the correlation between BR and contents of C and N in the soil is negative (R = -0.43 and -0.63). Thus, these values indicate that the respiratory activity of microorganisms decreases with the increasing C and N content in the soil. On the contrary, analysing the relationship between BR and C/N, revealed a positive correlation (R = 0.62). Spohn [95] also reached similar results in TN and C/N values. In his study, the relation between the content of N in the soil, C/N and BR was clearly identified. The increasing content of N substances resulted in the decreasing BR and, conversely, the increasing ratio of C and N was increasing BR. This phenomenon would also explain BR reduction in the case of digestate application, because digestates generally have lower C/N ratios and their application leads to the reduction of C/N ratio in the soil [92]. A decrease in the C/N value subsequently leads to a decrease in BR. The reason is a higher content of N substances in the digestate. The negative effect of TN on BR can be explained by the decomposition of SOM by microorganisms to obtain N. They also catabolize the easily available C to obtain energy for the SOM decomposition. This increases the presence of N in the soil but decreases the content of C and over time also BR, which gradually depletes metabolizable C content and increases the content of N which remains in the soil [95, 96]. In our experiment, a negative correlation between BR and TC was found. Although, this finding contradicts to the mentioned papers, this situation could have been caused by the application of digestate with a higher content of organic matter better resistant to degradation (CF, ADL). Thus, TC could not have been increased in the soil, except the maize + lupine variant (Fig. 3H). However, this variant showed the lowest BR, probably due to the higher N content in the available form.

No significant dependences were found between the other parameters, i.e., plant biomass production and soil parameters.

Conclusions

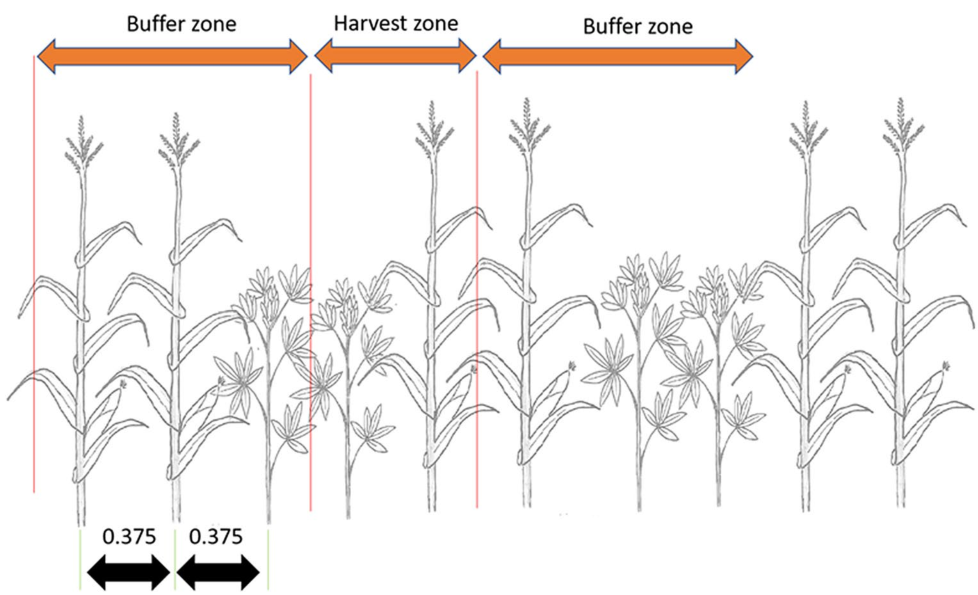
The first hypothesis was confirmed. Compared to digestate from the maize monoculture, the white lupine digestate showed the highest P and TN contents and and the maize + white lupine digestate was the most K abundant. The legumes were proven to increase the quality of digestate when used as a source of biomass for biogas production. On the other hand, the maize digestate contained also the highest CF (which represent less degradable substances), whereas the NDF content was increased in AD of maize with broad bean, in comparison to the maize monoculture. High NDF values indicated the presence of both complex (cellulose, lignin) and simpler carbohydrates, which could lead to higher digestibility. Furthermore, a potential of silage made of leguminous plants to increase N content in the digestate was found. Digestate, as an organic-mineral fertilizer, has a low C/N ratio which contributes to the rapid N release. Thus, it is desirable to increase the C/N ratio by raising the amount of C containing substances. This phenomenon must be further studied and described.

The second hypothesis was partly confirmed too. Significant differences were found between the effects of mixed and legume-based digestate types and the maize monoculture-derived type on the soil biology, albeit the changes were not all beneficial. The application of all digestate variants led to the reduction or unchanged BR and SIR values. The highest BR values were recorded in variants amended with digestates which contained increased DM and ADF contents: maize + broad bean and white lupine. The lowest values were found in the maize + white lupine variant, which also showed the highest content of C and N in the soil and led to the highest yield of plant biomass.

The third hypothesis was corroborated as well. All variants fertilized with digestate showed a statistically significant increase in plant biomass compared to the control without fertilization. However, statistically significant differences between the individual digestates were not observed due to a large variance of the measured values. The average calculated AGB values were difference between the variant with the application of digestate only from the maize silage (0.53 g·plant^{-1}) and the variants prepared from the mixed culture (+ 14%, i.e., 0.6 g·plant^{-1} in the maize + broad bean variant and + 33%, i.e., 0.7 g·plant^{-1} in the maize + white lupine variant). The potential for the addition of leguminous species to silage was obvious, especially the contribution of nutrients to arable land. Nevertheless, further tests must be conducted in the future.

Appendix

Appendix A: Biomass sampling for the preparation of model silages



Appendix B: Descriptive statistics for the chemical composition of used digestate—nutrient contents

See Tables 8 and 9

Table 8 Without the dependence of treatments

Treatments	Mean	SEM	SD	Min.	95%CI (Lower)	95%CI (Upper)	Max.
Dry matter (%)	2.79	0.05	0.21	2.37	2.65	3.00	3.17
P (g·kg^{-1})	26.38	1.22	4.73	21.01	22.46	32.31	33.00
K (g·kg^{-1})	8.57	0.36	1.42	6.16	7.14	9.67	10.71
N (g·kg^{-1})	165.93	4.39	17.03	141.30	149.66	184.74	193.33

Table 9 With the dependence of treatments

Parameter	Treatments	Mean	SEM	SD	Min.	Max.
Dry matter (%)	Maize	2.76	0.056862	0.09849	2.65	2.84
	Broad bean	2.49	0.064291	0.11136	2.37	2.59
	White lupine	3.00	0.111505	0.19313	2.79	3.17
	Maize + broad bean	2.94	0.075498	0.13077	2.79	3.03
	Maize + white lupine	2.80	0.049329	0.085440	2.72	2.89
P ($g \cdot kg^{-1}$)	Maize	22.10	0.96193	0.555368	21.01	22.83
	Broad bean	23.29	0.614284	1.06397	22.49	24.50
	White lupine	32.00	0.840417	1.45564	30.33	33.00
	Maize + broad bean	31.63	0.855648	1.48203	29.93	32.65
	Maize + white lupine	22.86	0.716667	1.241303	22.14	24.29
K ($g \cdot kg^{-1}$)	Maize	6.52	0.207846	0.36000	6.16	6.88
	Broad bean	8.83	0.403333	0.69859	8.43	9.64
	White lupine	9.67	0.193420	0.33501	9.33	10.00
	Maize + broad bean	7.82	0.340000	0.58890	7.14	8.16
	Maize + white lupine	10.00	0.544457	0.943027	8.93	10.71
TN ($g \cdot kg^{-1}$)	Maize	152.17	7.544438	13.06735	141.30	166.67
	Broad bean	180.72	8.359490	14.47906	164.66	192.77
	White lupine	183.33	6.938353	12.01758	170.00	193.33
	Maize + broad bean	159.86	5.891860	10.20500	149.66	170.07
	Maize + white lupine	153.57	4.122281	7.140000	146.43	160.71

Appendix C: Descriptive statistics for the chemical composition of used digestate—content of organic compounds

See Tables 10 and 11

Table 10 Without the dependence of treatments

Treatments	Mean	SEM	SD	Min.	95% CI (Lower)	95% CI (Upper)	Max.
CF ($\%_{TS}$)	7.94	0.51	1.99	5.33	6.12	9.64	11.59
ADF ($\%_{TS}$)	18.11	1.32	5.13	10.71	14.86	23.81	25.33
NDF ($\%_{TS}$)	25.92	1.30	5.03	16.79	21.01	29.93	32.33
ADL ($\%_{TS}$)	9.87	0.70	2.72	6.79	7.50	11.24	15.00

Table 11 With the dependence of treatments

Parameter	Treatments	Mean	SEM	SD	Min.	Max.
CF ($\%_{TS}$)	Maize	10.87	0.42	0.73	10.14	11.59
	Broad bean	9.24	0.40	0.70	8.43	9.64
	White lupine	5.67	0.19	0.34	5.33	6.00
	Maize + broad bean	6.80	0.34	0.59	6.12	7.14
	Maize + white lupine	7.14	0.20	0.36	6.79	7.50
ADF ($\%_{TS}$)	Maize	15.58	0.42	0.72	16.30	14.86
	Broad bean	16.06	0.46	0.81	15.26	16.87
	White lupine	24.00	0.77	1.33	22.67	25.33
	Maize + broad bean	23.47	0.90	1.56	21.77	24.83
	Maize + white lupine	11.43	0.41	0.72	10.71	12.14
NDF ($\%_{TS}$)	Maize	22.82	0.91	1.58	23.91	21.01
	Broad bean	29.32	0.93	1.61	27.71	30.92
	White lupine	30.67	0.84	1.45	29.67	32.33
	Maize + broad bean	28.57	1.04	1.80	26.53	29.93
	Maize + white lupine	18.21	0.71	1.23	16.79	18.93
ADL ($\%_{TS}$)	Maize	7.49	0.32	0.56	7.97	6.88
	broad bean	10.31	0.48	0.83	9.64	11.24
	White lupine	14.33	0.38	0.67	13.67	15.00
	Maize + broad bean	10.09	0.30	0.52	9.52	10.54
	Maize + white lupine	7.14	0.20	0.36	6.79	7.50
TC ($\%_{TS}$)	Maize	29.33	0.010	0.017896	29.31150	29.34600
	Broad bean	28.91	0.042	0.072187	28.83700	28.98100
	White lupine	28.85	0.038	0.065000	28.78000	28.91000
	Maize + broad bean	29.64	0.031	0.053266	29.60600	29.69900
	Maize + white lupine	29.58	0.006	0.011034	29.56600	29.58800
TOC ($\%_{TS}$)	Maize	29.25	0.007	0.011790	29.24200	29.26500
	Broad bean	28.80	0.039	0.067639	28.73900	28.87400
	White lupine	28.74	0.035	0.061000	28.68000	28.80100
	Maize + broad bean	29.54	0.031	0.053388	29.50000	29.60100
	Maize + white lupine	29.45	0.010	0.017755	29.44400	29.47500

CF crude fibre, *ADF* acid detergent fibre, *NDF* neutral detergent fibre, *ADL* acid detergent lignin, *TC* total carbon, *TOC* total organic carbon

Appendix D: Overview of nutrient inputs by the application of individual types of digestate

See Tables 12 and 13

Table 12 Input of P, K and N in kg·ha^{-1}

Treatments	P		K		N	
	kg·ha^{-1}	± SE	kg·ha^{-1}	± SE	kg·ha^{-1}	± SE
M	20.75	0.52^{c}	6.12	0.20	142.86	7.08
B	18.41	0.48^{c}	6.98	0.32	142.86	6.61
L	24.94	0.65^{b}	7.53	0.15	142.86	5.41
MB	28.27	0.76^{a}	6.99	0.30	142.86	5.26
ML	21.26	0.66^{c}	9.30	0.51	142.86	3.84

M maize, *B* broad bean, *L* white lupine, *MB* maize + broad bean, *ML* maize + white lupine

Average values ($n = 3$; ± SE) of individual nutrient doses are displayed

The doses of nutrients are converted to dry matter per 1 ha

Different letters indicate statistically significant differences ($p \leq 0.05$) between individual variants in the content of selected parameter

Table 13 Input of CF, ADF, NDF and ADL in kg·ha^{-1}

Treatments	CF		ADF		NDF		ADL	
	kg·ha^{-1}	±SE	kg·ha^{-1}	±SE	kg·ha^{-1}	±SE	kg·ha^{-1}	±SE
M	102.04	3.93	146.26	3.93	214.29	8.56	70.29	3.00
B	73.02	3.17	126.98	3.67	231.75	7.33	81.48	3.82
L	44.16	1.50	187.01	6.00	238.96	6.54	111.69	3.00
MB	60.79	3.04	209.73	8.04	255.32	9.29	90.17	2.68
ML	66.45	1.92	106.31	3.84	169.44	6.64	66.45	1.92

M maize, *B* broad bean, *L* white lupine, *MB* maize + broad bean, *ML* maize + white lupine

Average values ($n = 3$; ±SE) of organic matter doses are displayed CF (crude fibre). ADF (acid detergent fibre). NDF (neutral detergent fibre). ADL (acid detergent lignin) in individual variants of digestate

The doses are converted to dry matter per 1 ha

Different letters indicate statistically significant differences ($p \leq 0.05$) between individual variants in the content of selected parameter

Appendix E

See Table 14

Table 14 Results of the one-way analysis of variance (ANOVA)

Soil properties		F test	Significance (p)
Respiration	Basal respiration	F (5.90) = 22.80	< 0.001***
	Alanine	F (5.90) = 19.15	< 0.001***
	Lysine	F (5.90) = 21.48	< 0.001***
	Glucosamine	F (5.90) = 20.25	< 0.001***
	Glucose	F (5.90) = 16.48	< 0.001***
	Trehalose	F (5.90) = 14.14	< 0.001***
Biomass	Total dry	F (5.42) = 9.17	< 0.001***
Chemical element	Total carbon	F (5.42) = 11.29	< 0.001***
	Total nitrogen	F (5.42) = 21.97	< 0.001***
	C/N ratio	F (5.42) = 14.50	< 0.001***

*statistically significant difference at a 5% significance level

**statistically significant difference at a 1% significance level

***statistically significant difference at a 0.01% significance level

Appendix F

See Tables 15 and 16

Table 15 Descriptive statistics for basal and substrate-induced respiration with the dependence of treatments

Respiration	Treatments	Mean	SEM	SD	Min.	95%CI (Lower)	95%CI (Upper)	Max.
Basal	Negative control	0.28	0.01	0.05	0.21	0.26	0.31	0.38
	Maize	0.19	0.01	0.04	0.16	0.17	0.21	0.26
	Broad bean	0.21	0.01	0.02	0.16	0.20	0.22	0.25
	White lupine	0.22	0.01	0.05	0.17	0.20	0.25	0.31
	Maize + broad bean	0.24	0.00	0.02	0.23	0.24	0.25	0.27
	Maize + white lupine	0.16	0.01	0.02	0.12	0.15	0.17	0.23
Alanine	Negative control	0.39	0.02	0.06	0.24	0.35	0.42	0.47
	Maize	0.29	0.02	0.06	0.22	0.25	0.32	0.46
	Broad bean	0.39	0.01	0.05	0.29	0.37	0.41	0.47
	White lupine	0.32	0.02	0.07	0.20	0.28	0.36	0.43
	Maize + broad bean	0.32	0.01	0.06	0.23	0.29	0.35	0.40
	Maize + white lupine	0.23	0.01	0.03	0.16	0.21	0.24	0.30
Lysine	Negative control	0.29	0.01	0.05	0.19	0.27	0.32	0.35
	Maize	0.21	0.01	0.04	0.16	0.19	0.23	0.32
	Broad bean	0.26	0.01	0.03	0.18	0.24	0.27	0.30
	White lupine	0.23	0.01	0.05	0.17	0.21	0.26	0.28
	Maize + broad bean	0.26	0.01	0.03	0.21	0.24	0.27	0.29
	Maize + white lupine	0.17	0.01	0.02	0.14	0.16	0.18	0.21
Glucosamine	Negative control	0.39	0.01	0.06	0.27	0.36	0.42	0.47
	Maize	0.27	0.01	0.03	0.23	0.25	0.28	0.35
	Broad bean	0.34	0.01	0.06	0.24	0.31	0.37	0.41
	White lupine	0.30	0.02	0.07	0.20	0.27	0.34	0.43
	Maize + broad bean	0.32	0.01	0.04	0.25	0.30	0.34	0.41
	Maize + white lupine	0.22	0.01	0.04	0.18	0.20	0.24	0.29
Glucose	Negative control	0.69	0.03	0.10	0.45	0.64	0.75	0.82
	Maize	0.45	0.01	0.06	0.37	0.42	0.48	0.60
	Broad bean	0.76	0.04	0.14	0.51	0.68	0.83	0.97
	White lupine	0.53	0.04	0.15	0.26	0.46	0.61	0.73
	Maize + broad bean	0.71	0.02	0.07	0.61	0.67	0.75	0.85
	Maize + white lupine	0.48	0.05	0.20	0.21	0.37	0.58	0.86
Trehalose	Negative control	0.53	0.02	0.08	0.36	0.49	0.58	0.65
	Maize	0.36	0.01	0.03	0.32	0.35	0.38	0.48
	Broad bean	0.50	0.02	0.06	0.36	0.46	0.53	0.57
	White lupine	0.42	0.03	0.11	0.27	0.36	0.48	0.65
	Maize + broad bean	0.44	0.01	0.04	0.38	0.42	0.46	0.53
	Maize + white lupine	0.34	0.03	0.11	0.18	0.28	0.40	0.56

Appendix G

See Table 16

Table 16 Descriptive statistics for basic soil properties with the dependence of treatments

Soil properties	Treatments	Mean	SEM	SD	Min.	95%CI (Lower)	95%CI (Upper)	Max.
Total carbon	Negative control	0.78	0.01	0.03	0.75	0.75	0.80	0.82
	Maize	0.77	0.01	0.02	0.74	0.75	0.78	0.79
	Broad bean	0.77	0.01	0.03	0.73	0.75	0.80	0.83
	White lupine	0.77	0.01	0.03	0.72	0.74	0.79	0.80
	Maize + broad bean	0.79	0.01	0.04	0.71	0.76	0.83	0.83
	Maize + white lupine	0.86	0.01	0.03	0.80	0.83	0.89	0.90
Total nitrogen	Negative control	0.07	0.00	0.01	0.06	0.06	0.07	0.08
	Maize	0.07	0.00	0.01	0.06	0.07	0.08	0.08
	Broad bean	0.08	0.00	0.00	0.07	0.08	0.08	0.08
	White lupine	0.07	0.00	0.00	0.06	0.06	0.07	0.07
	Maize + broad bean	0.07	0.00	0.00	0.07	0.07	0.08	0.08
	Maize + white lupine	0.09	0.00	0.00	0.08	0.09	0.09	0.10
C/N ratio	Negative control	11.43	0.24	0.67	10.28	10.87	11.98	12.27
	Maize	10.70	0.30	0.86	9.30	9.98	11.41	11.65
	Broad bean	9.98	0.07	0.21	9.79	9.80	10.15	10.37
	White lupine	11.22	0.16	0.46	10.59	10.84	11.61	11.83
	Maize + broad bean	10.79	0.10	0.29	10.46	10.55	11.04	11.18
	Maize + white lupine	9.65	0.09	0.26	9.25	9.43	9.87	10.09
Total dry	Negative control	0.35	0.02	0.05	0.31	0.31	0.40	0.44
	Maize	0.57	0.07	0.20	0.41	0.41	0.74	0.87
	Broad bean	0.65	0.03	0.08	0.58	0.59	0.72	0.77
	White lupine	0.72	0.05	0.15	0.52	0.60	0.85	0.92
	Maize + broad bean	0.65	0.02	0.05	0.58	0.61	0.70	0.70
	Maize + white lupine	0.76	0.07	0.19	0.53	0.60	0.91	1.02

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Author contributions

MB was involved in conceptualization, formal analysis, investigation, writing—original draft, project administration; AK, JH, TH were involved in conceptualization, writing—review and editing, resources; AM, JK and JP were involved in writing—review and editing, TV was involved in data curation, resources; TB was involved in data curation and visualization; JE was involved in supervision, conceptualization, writing—review and editing, investigation and validation. All authors read and approved the final manuscript.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate

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Consent for publication

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Competing interests

The authors declare no competing interests.

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