



Investigating the usability of black locust (*Robinia pseudoacacia* L.) cultivars for feed purposes in Hungary

Rita Tömösközi-Farkas[✉] · Éva Lengyel-Kónya[✉] · Mária Berki[✉] · Zsolt Zalán[✉] · Krisztina Takács[✉] · Emőke Szerdahelyi[✉] · Enikő Horváth-Szancsics · Nóra Adányi[✉] · Attila Borovics[✉] · János Rásó[✉] · Klára Cseke[✉] · Tamás Molnár[✉] · Zsolt Keserű[✉]

Received: 12 March 2026 / Accepted: 21 May 2026 / Published online: 30 May 2026
© The Author(s) 2026

Abstract The increasing demand for protein in animal husbandry necessitates the identification of alternative feed sources to partially replace soybean meal. Black locust (*Robinia pseudoacacia* L.), one of the most widespread tree species in Hungary, represents a promising candidate due to its high biomass production and potential nutritional value. The aim of this study was to evaluate the compositional characteristics and fermentation potential of leaves and pods from selected black locust varieties, in comparison with alfalfa as a reference feed. The chemical composition (dry matter, crude protein, fat, ash), total polyphenol content (Folin-method), polyphenol profile (HPLC), amino acid profile (HPLC), lipid profile (GC), antioxidant capacity (TEAC method), and the presence of antinutritional proteins were determined (PAGE). In addition, the lactic fermentation potential of the plant

materials was assessed. Comparing the nutritional data of black locust to alfalfa (Blume et al. 2021), we found that the dry matter and protein content of the two feeds are similar (92%; 17%), while the fat content (2.8%—alfalfa; 4.27%—black locust) of black locust is higher and the ash content (14.6%—alfalfa; 6.1%—black locust) is lower. It should be emphasized that the total polyphenol content (30,000 mg GAE/kg—black locust; 6171 mg GAE/kg—alfalfa) and antioxidant capacity (225.71 mM Tr/kg—black locust; 9.6 mg mM Tr/kg—alfalfa) of black locust foliage are also significantly higher than that of alfalfa. Our experimental results showed that black locust leaves are lacto-fermentable, which may increase the digestibility and nutritional value of feeds. Antinutritional proteins such as Kunitz-type trypsin inhibitors or Bowman–Birk-type trypsin and chymotrypsin inhibitors could only be detected in the pod samples, not in the foliage, so they do not affect digestibility of foliage. These results indicate that black locust foliage and to a lesser extent the pods may serve as a valuable complementary protein source in animal feeding.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10457-026-01553-0>) contains supplementary material, which is available to authorized users.

R. Tömösközi-Farkas (✉) · É. Lengyel-Kónya · M. Berki · Z. Zalán · K. Takács · E. Szerdahelyi · E. Horváth-Szancsics · N. Adányi
Institute of Food Science and Technology, Hungarian University of Agriculture and Life Sciences, Villányi Str. 29-43, Budapest 1118, Hungary
e-mail: Tomoskozine.Farkas.Rita.Adel@uni-mate.hu

A. Borovics · J. Rásó · K. Cseke · T. Molnár · Z. Keserű
Forest Research Institute, University of Sopron, Várkerület 30/A, Sárvár 9600, Hungary

Keywords Black locust · Feed · Cultivars · Microbiological tests · Genetic tests

Introduction

Black locust (*Robinia pseudoacacia* L.) is an economically important, vital, fast-growing, stand-forming

tree species in Hungary, and its importance is increasing in many other countries. The species is indigenous to the eastern and central parts of the USA between latitudes 35° and 43° N. Black locust is a typical multipurpose tree species with various uses, including timber and commercial honey production, and is suitable for ornamental purposes. Besides these features, the species is remarkably resilient against biotic threats, including pests and diseases, and is susceptible only to frost damage, particularly late-spring frosts and hard winter frosts (Keresztesi 1988). Black locust is Hungary's most widespread tree species, occupying approximately 24% of the forested land (465,000 ha) and providing 25% of the annual timber output. The mean wood volume across all black locust forests is 125 m³/ha, increasing to 190 m³/ha at the final cutting age (31 years on average). Moreover, about 40% of black locust stands in Hungary grow under marginal site conditions in the lowlands with a forest-steppe climate, where annual precipitation is less than 500 mm, most of it falling outside the growing season, and where summer droughts coupled with high temperatures are common.

In Hungary, the rapid spread of black locust was promoted both in the past and in the present by the following factors: (1) anchoring loose, shifting sand; (2) afforesting abandoned agricultural lands; (3) obtaining abundant seed yield, vitality, the excellent ability for sprouting and ability to fix free atmospheric nitrogen; (4) utilizing a broad range wood uses (tool manufacturing, fuel wood, vineyard applications, poles and props, pit-wood, parquet flooring); and (5) encouraging bee foraging for honey production.

The Hungarian Forest Research Institute has been engaged in resolving various black locust management issues, and several research findings have been implemented in practice (Rédei et al. 2017). Clone selection and tree improvement also have a long tradition, primarily for wood production purposes (Ábri et al. 2023). In apiculture research, in addition to honey production (Keresztesi 1983; Farkas and Zajác 2007; Samsonova et al. 2020) and the nectar characteristics of selected genotypes (Kim et al. 2020), pollination ability (Carl et al. 2017; Giovanetti et al. 2018) is a highlighted topic nowadays.

In the nineteenth and twentieth centuries, foliar fodder was regularly collected in Hungary (Varga 2017). The increase in drought years has renewed interest in foliar fodder across the

country, particularly in black locust, which can grow in extreme conditions where other protein crops struggle or fail, thus providing a realistic alternative in drying climatic conditions. According to the relevant international literature (Zang et al. 2010), the crude protein content of black locust foliage is close to that of alfalfa (24%). However, the digestibility of black locust leaves is lower, according to some literature sources (Chen et al. 2011). Therefore, it can be a vital supplementary feed. Livestock can also access other valuable plants in black locust forest stands and plantations. Moreover, naturally arising polyploid forms of *Robinia*, plants possessing more than two chromosome sets compared with a diploid individual due to a genome multiplication event, can also be of interest from a feedstock biomass production perspective (Keresztesi 1984). Higher biomass production or yield can be a common consequence of polyploidization due to larger leaf size or increased vigor in general (Comai 2005; Corneillie et al. 2019).

There have been several publications on the toxicity and therapeutic effects of *Robinia*. Black locust contains numerous toxic components in its leaves, stems, bark, and seeds, and their effects can cause gastrointestinal and neurological problems, as well as tissue necrosis (Rabani and Melzig 2010; Browne et al. 2020; Móricz et al. 2024) in various animals and humans. The two main toxalbumins, robin and phasin, exert their toxic effects by inhibiting protein synthesis (Hui et al. 2024). Hsu et al. (2004) showed that acacetin, a flavonoid (5,7-dihydroxy-40-methoxyflavone) found in black locust leaves, inhibits cell growth, induces cell cycle arrest, and plays a role in inducing apoptosis (Zarev 2023). Burner et al. (2007) suggest that black locust is toxic for horses but not goats. The toxicity to cattle and sheep remains unclear and has not been adequately demonstrated. Data on the effect of black locusts on ruminants are limited or even contradictory. On the other hand, studies also suggest that black locust foliage promotes antler growth in deer (Halász et al. 2021; Snyder et al. 2006) and is an excellent fodder plant for pig fattening and chicken rearing (Papachristou 1999).

The present study aims to examine the chemical content and digestibility of leaves and pods from different Hungarian black locust varieties with versatile aspects. A further aim of our research is to perform DNA fingerprinting using nuclear simple sequence repeat (nSSR) markers, along with chemical surveys,

to verify the clonal identity and ploidy level of the varieties selected for the study, as these factors affect biomass production.

Material and methods

Plant material

Foliage and pod samples from black locust cultivars were collected from two study plots in the Hungarian Great Plain in 2019, one in subcompartment Püspökladány 23/O (coord.: E 804337, N 223466) and the other in Hajdúhadház 16/D (coord.: E 850098, N 260855). Table 1 lists the varieties included in the analysis. Eleven different cultivars were evaluated in the foliage analysis, of which two clones can be found in both trials. The number of repetitions was three individuals per clone. Leaves were harvested on September 12, 2019, in Hajdúhadház and on September 18, 2019, in Püspökladány. Leaf samples were collected together with the petiole from all parts of the canopy (lower, middle, and upper parts) and mixed. Pod analyses

were collected from 10 cultivars at one study site, with three replicates per cultivar. Pods were collected on September 18, 2019, in Püspökladány. Pods were harvested from all parts of the canopy, including seeds. The seeds were not yet fully mature, but they were close to full maturity. Black locust seeds typically reach full maturity in October.

Basic compositional analyses, including determination of dry matter, crude protein, crude fat, ash content, total polyphenol concentration, and anti-oxidant capacity, were carried out on all analyzed black locust leaf and pod samples.

For both leaf and pod samples, three biological replicates were analyzed. Each replicate consisted of a composite sample from a single tree: leaves or fruits were collected, pooled, and homogenized, and the resulting homogenate was used for subsequent analyses. Thus, three independent homogenized samples originating from three different trees were prepared, and each was subjected to a single analytical determination and fermentation experiment.

In the case of alfalfa, three independently prepared samples were obtained from the same bulk material, and each sample was analyzed once under identical conditions.

For subsequent detailed investigations, two pod samples (ET01 and ET06) were selected. Both samples originated from the same growing site but represented different cultivars (Table 1). These pods were chosen because sufficient quantities were available to perform the full range of analyses, including chemical characterization and lactic acid fermentation experiments.

From the leaf material, five samples (EL01, EL06, EL07, EL11, and EL13) were selected for further study. Among these, two leaf samples corresponded to the selected pod samples (EL01–ET01 and EL06–ET06), while the remaining samples originated from different cultivars and growing sites.

For the in vitro digestion experiments, two leaf and two pod samples originating from the same trees were selected (EL01–ET01 and EL06–ET06). This paired sampling strategy was applied to minimize biological variability among individual trees and to enable a more reliable comparison of the digestion behavior of leaf and fruit matrices.

Based on the total polyphenol measurements, the detailed polyphenol profile analysis was performed

Table 1 The black locust cultivars included in the chemical analysis

Sample ID	Sample type	Cultivar	Study site
EL1	Foliage	Üllői	Püspökladány
EL2	Foliage	PL40	Püspökladány
EL3	Foliage	Zalai	Püspökladány
EL4	Foliage	Debreceni-2	Püspökladány
EL5	Foliage	PL324	Püspökladány
EL6	Foliage	PL251	Püspökladány
EL7	Foliage	Szajki	Püspökladány
EL8	Foliage	Váti	Püspökladány
EL9	Foliage	PL35	Püspökladány
EL10	Foliage	Nyírségi	Püspökladány
EL11	Foliage	Üllői	Hajdúhadház
EL12	Foliage	Kiskunsági	Hajdúhadház
EL13	Foliage	Szajki	Hajdúhadház
ET1	Pods	Üllői	Püspökladány
ET3	Pods	Zalai	Püspökladány
ET4	Pods	Debreceni-2	Püspökladány
ET6	Pods	PL251	Püspökladány
ET8	Pods	Váti	Püspökladány
ET9	Pods	PL35	Püspökladány

exclusively on leaf samples, as the fruits contained an order-of-magnitude lower amount of phenolic compounds. In addition to the five samples originally selected for further analyses, two additional samples (EL09 and EL10) were included due to their high total polyphenol content and antioxidant capacity values (Table 2).

The alfalfa pellet used as a control consisted of 100% alfalfa and was obtained from Fonolit Ltd. (Szeged, Hungary).

Method of the SSR analysis

Total genomic DNA was extracted from fresh leaves using the ATMAb procedure, a modified cetyltrimethylammonium bromide (CTAB) method (Dumolin et al. 1995). DNA concentration was measured by spectrophotometry (Eppendorf BioPhotometer, Hamburg, Germany) and standardized to 10 ng/μL. A total

of 10 SSR markers were used for genetic fingerprinting based on Lian and Hogetsu (2002) and Mishima et al. (2009) and assembled in two marker sets as the following (5' Forward fluorescent dyes in brackets): Set1—Rops 02 (6-FAM), Rops 05 (VIC), Rops 18 (NED), Rops 09 (PET), Rops 08 (6-FAM); Set2—Rops 16 (FAM), Rops 150 (NED), Rops 109 (NED), Rops 200 (VIC), Rops 206 (VIC). The PCR was performed using the Type-it Microsatellite PCR Kit by QIAGEN (Hilden, Germany) following the manufacturer's instructions in a Veriti Personal Thermocycler (Applied Biosystems, Foster City, California, USA). Amplification products were diluted 20-fold for capillary electrophoresis and denatured in formamide (Hi-Di, Applied Biosystems, Waltham, Massachusetts, USA) using the GeneScan LIZ 500 (Applied Biosystems, Waltham, Massachusetts, USA) internal size standard. SSR genotyping was performed on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster

Table 2 Main composition, antioxidant capacity, and total polyphenol content of black locust foliage (EL), pod samples (ET), and alfalfa pellets

Samples	Dry matter (%)		Crude protein content (%)		Fat content (%)		Ash content (%)		Total polyphenol (mg GAE/kg)		Antioxidant capacity, DPPH (mM Tr/kg)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
EL01	93.9 b	0.3	17.9 c	0.3	6.0 b	0.1	8.3 d	0.2	20,436 d	171	149.9 f	9.7
EL02	94.8 a	0.1	17.3 c, d	0.4	6.9 a	0.1	10.3 c	0.1	20,264 d	312	112.0 g	2.2
EL03	94.7 a	0.4	16.8 d	0.1	6.5 a,b	0.4	11.2 b	0.1	16,230 e	705	103.8 g	8.9
EL04	94.9 a	0.1	15.5 f	0.1	5.3 c	0.3	6.5 h	0.1	37,593 a	1913	263.7 c	16.4
EL05	93.6 b	0.4	16.8 d	0.2	4.6 d	0.3	5.7 i	0.5	41,755 a	1759	334.3 b	8.5
EL06	94.1 b	0.3	20.0 a	0.2	4.5 d	0.2	8.5d	0.2	26,066 c	1746	182.9 e	11.0
EL07	94.1 b	0.3	20.0 a	0.1	4.5 d	0.3	8.5 d	0.1	29,979 b	1454	231.6 c	8.3
EL08	94.1 b	0.2	19.2 b	0.0	4.1 d	0.2	7.2 f	0.1	29,610 b	1315	203.9 d	6.6
EL09	92.6 c	0.1	16.8 d	0.2	4.8 c,d	0.5	7.6 e	0.1	37,108 a	1563	316.0 b	18.5
EL10	94.6 a	0.2	17.0 d	0.2	4.2 d	0.1	8.7 d	0.1	41,439 a	1642	359.0 a	8.5
EL11	94.0 b	0.2	19.3 b	0.1	4.5 d	0.0	6.9 g	0.0	25,628 b,c	1843	169.9 e,f	9.6
EL12	93.8 b	0.3	18.2 c	0.1	5.9 b	0.2	8.7 d	0.1	16,490 e	543	71.4 h	4.2
EL13	93.7 b	0.2	19.2 b	0.1	5.5 c	0.1	7.3 f	0.1	17,349 e	637	78.9 h	7.1
ET01	89.6 e	0.3	19.2 b	0.2	3.3 e	0.0	3.1 k	0.2	7415 f	591	30.2 h	0.8
ET03	90.1 d,e	0.4	18.4 c	0.2	3.4 e	0.1	2.9 k	0.0	6608 f,g	601	26.6 i	2.2
ET04	90.4 d	0.1	15.7 f	0.1	2.7 f	0.1	3.1 j,k	0.4	6294 g	269	26.1 i	2.0
ET06	90.3 d,e	0.7	17.8 c	0.1	4.2 d	0.1	3.7 j	0.1	5609 g	362	23.3 i	2.4
ET08	89.1 d,e	0.7	18.3 c	0.3	3.5 e	0.1	3.6 j	0.1	5788 g	451	23.9 i	2.9
ET09	89.4 e	0.5	16.1 e	0.1	2.7 f	0.0	3.7 j	0.0	4104 h	61	15.4 j	2.0
Alfalfa pellets	92.4 c	0.3	17.0 d	0.0	2.8 f	0.3	14.6 a	0.3	6171 g	175	9.6 k	0.5

Different letters indicate statistically significant differences within columns according to Tukey's multiple range test ($p < 0.05$)

City, California, USA) at Biomi Ltd. (Gödöllő, Hungary). Allele calling was conducted using GeneMapper 4.0 software (Applied Biosystems, Foster City, California, USA).

GenAlEx 6.5 software (Peakall and Smouse 2006, 2012) was used to analyze SSR data. The probability of identity (PI) was calculated from the 10 analyzed loci, and possible matching genotypes were assessed. The genetic distance (Codom-Genotypic) option was applied to generate a pairwise, individual-by-individual genetic distance matrix. The matrix was used for subsequent PCoA and for constructing the UPGMA dendrogram by PAST 4.03 (Hammer et al. 2001). In PCoA analysis, the ordination axes summarize overall genetic dissimilarities among genotypes rather than individual traits; therefore, the results are interpreted based on the proportion of variance explained by each axis.

Another six genotypes were added to the 11 varieties under investigation to test ploidy levels. These genotypes were collected from a former experimental plot of the Sárvár Experimental Station of the Hungarian Forest Research Institute. The 54-year-old experimental plot is no longer under investigation, and high mortality has occurred due to the age of the plant material. Based on the 2014 condition survey, only six elderly tree individuals were accurately identified as putative polyploids. Unfortunately, the origin of the plant material is unknown, and the identification codes do not match those reported in the literature (Keresztesi et al. 1984). Nevertheless, the following six genotypes were included in the genetic analysis to assess ploidy levels and potential polyploidy: 'K62', '4', 'K68', 'I1', 'K66', and 'F1'.

Determination of the main composition

Moisture content was measured according to the ISO 6496 (1999) at 103 °C (± 2 °C) for six hours at atmospheric pressure. Oil content was extracted with petrol ether according to ISO 6492 (1999). Ash content was determined by ashing at 550 °C until a constant weight was obtained (ISO 2171:2023). Crude protein content was determined by the AOAC Method No. 968.06 (2003).

Following a general examination of the chemical composition, we also performed a more detailed

analysis of selected cultivars, examining the effects of place of origin and the composition of foliage and pod samples from the same cultivars. EL1 and EL11, as well as EL7 and EL13, were foliage samples from the same cultivar but originated from separate places, Püspökladány and Hajdúhadház, respectively. EL1 and ET1, as well as EL6 and ET6, were foliage and pod samples from the same cultivars and origin. In addition to the main components, the amino acid and fatty acid profiles were determined in the selected samples. The protein patterns and potentially present antinutritional proteins were examined and supplemented with in vitro digestion experiments. The effect of lactic acid fermentation, which improves digestibility, was also examined in the selected samples.

Amino acid analysis

Acid hydrolysis was performed for amino acid analysis. Twenty-five milligrams of the samples were hydrolyzed by 6 N HCl containing 1% phenol in a Milestone ETHOS One Microwave digestion system. Hydrolysates were completed to 5 mL with borate buffer (1 M, pH 8.51).

Derivatization was performed following the Waters AccQTag method. Waters Acquity UPLC H-Class equipment was used with a PDA detector at 260 nm for the measurements. The column was AccQ UPLC BEH C18 2.1 $\times$ 100 mm, 1.7 μ m (eluent: AccQ Tag Ultra eluent A, B, water) in gradient mode. The column temperature was 43 °C, the flow rate was 0.7 mL/min, and the injection volume was 10 μ L.

Determination of antioxidant capacity

Antioxidant capacity was determined according to Brand-Williams et al. (1995). Samples were extracted with methanol, stored at 4 °C for 24 h, and filtered after 30 min of shaking. The color reaction was conducted with 2,2-diphenyl-1-picrylhydrazyl at 36 °C for 30 min in a dark place, and the decrease of absorbance relative to a blank sample was measured at 517 nm (ATI Unicam UV-Vis Spectrometer UV2, Unicam, UK). The antioxidant capacity is expressed as Trolox equivalents per 100 g DM.

Determination of total polyphenol content

Total polyphenols were determined according to Singleton et al. (1999). Defatted samples were extracted with methanol, stored at 4 °C for 24 h, and filtered after 30 min shaking. A color reaction with the Folin–Ciocalteu reagent was performed, and absorbance was measured at 750 nm. The results are expressed as gallic acid equivalents per 100 g of dry matter (GAE/100 g DM).

Determination of fatty acid composition

Fatty acids were analyzed from the extracted oil by gas chromatography (Agilent 7890A GC System, Santa Clara, California, USA) as methyl esters of fatty acids (Tóth-Márkus and Sass-Kiss 1993). Ten milligrams of fat were saponified with 250 µL of 0.5 M KOH/methanol in a 2-mL screw-cap glass vial and heated for 15 min in a blocked thermostat at 140 °C. The transesterification was performed with boron trifluoride in methanol (14%, Sigma, Inc., USA) for 10 min, and then 250 µL of p.a. heptane (Merck, Germany) was added, and the mixture was brought to a boil. After the cooling period, up to 2 mL of saturated sodium chloride solution was added. After standing for 1 h, the separated upper phase was moved into a test vial containing a 1-mm layer of dry sodium sulfate, to which 0.5 mL of heptane was added. The column was a Supelco SP-2560 with film dimensions of 100 m×0.25 mm×0.2 µm (Supelco, USA). The oven temperature program was 5 min at 140 °C, followed by a ramp of 4 °C/min to 240 °C, with 10 min at the final temperature. The injector temperature was 220 °C, and the detector temperature was 250 °C. The carrier gas was hydrogen, with a column flow of 1 mL min⁻¹ and an automatic injection volume of 1 µL.

Determination of phenolic components

To prepare the samples, 0.02 g of defatted and ground leaves were weighed into a 2 mL Eppendorf tube and rehydrated with 0.5 mL of distilled water. Then, 1 mL of 2% acetic acid in methanol was added. The mixture was sonicated for 20 min at room temperature. After centrifugation (10,000 rpm, 10 min), the supernatant was poured into another tube, and the residue was re-extracted with 1 mL of 2% acetic acid in methanol.

The collected supernatants were filtered through a 0.45 µm PVDF syringe filter.

The pod extract was prepared similarly, but we measured 0.05 g samples and used a one-step extraction.

A Waters Alliance system (Waters, Milford, USA) consisting of a Model e2695 separation module with a Model 2998 photodiode array (PDA) detector run by Empower software (Waters, Milford, USA) was used for the determination of the phenolic compounds. The separation was performed on a Kinetex Biphenyl 5 µm, 150×4.6 mm column (Phenomenex, Torrance, USA) with gradient elution. The mobile phase was (A) 0.1% formic acid in bidistilled water and (B) 0.1% formic acid in acetonitrile.

Fermentation experiment of black locust samples with lactic acid bacteria

Five grams of black locust foliage/pod sample was weighed into 45 mL sterile RO water and, after vigorous stirring, inoculated with 1% *Lactobacillus* suspension. The applied starter strains were *Lactobacillus* (L.) *casei* Shirota and *Lactobacillus plantarum* 2142. The inoculated samples were incubated at 30 °C for 48 h. The viable *Lactobacillus* cell count was determined from the samples after serial decimal dilutions on selective MRS agar plates according to the Miles and Mishra method. The acidification was determined by pH measurements to monitor fermentation and metabolite production in the starter culture. The fermentation experiment was performed in duplicate. The changes in the protein profile were due to fermentation, and polyacrylamide gel electrophoresis (PAGE) was performed.

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE and Native PAGE were performed on 15% polyacrylamide gels with 6% stacking gels according to Laemmli protocol (Laemmli 1970). The vertical mini-PROTEAN 3 system (Bio-Rad, Hercules, California, USA) with a 0.75 mm spacer thickness was used. Separation was done at 200 V for about 40–55 min at room temperature. Separated proteins were stained with Coomassie Brilliant BlueR-250. Image analysis of gels was performed using the Bio-Rad Gel Doc 2000 system (Hercules, California, USA).

Determination of trypsin and chymotrypsin inhibitor activity on native PAGE gels

Native PAGE gels were stained for trypsin and chymotrypsin inhibitor activities, as described by Kolipara and Hymowitz (1992). Immediately after separation, the gel was incubated in a freshly prepared 0.25 mg/mL solution of bovine trypsin (Sigma T4665) or chymotrypsin (Fluka 27270) in 0.1 M phosphate buffer, pH 7.5, at 43 °C for 30 min. Inhibitory activity was visualized by staining the gel with tetrazotized o-dianisidine as a dye (Sigma A3502, Fast blue B salt) solution containing N-acetyl-DL-phenylalanine- β -naphthyl ester (Sigma A7512). Image analysis of gels was performed with the Bio-Rad Gel Doc 2000 system (Bio-Rad, Hercules, California, USA). The separated bands were compared to Kunitz-type trypsin inhibitor (KTI) and Bowman-Birk-type trypsin-chymotrypsin inhibitor (BBI) as major protease inhibitor types.

Simulated human in vitro gastrointestinal digestion

In vitro digestion was performed according to the procedure described by Minekus et al. (2014). Simulated salivary fluid (SSF, pH 7.0), simulated gastric fluid (SGF, pH 3.0), and simulated intestinal fluid (SIF, pH 7.0) were used. These were made of the appropriate electrolyte solutions, enzymes, CaCl_2 , and water. Enzyme activities were adjusted according to the assumable physiological circumstances and used as follows: human salivary α -amylase (Sigma A1031-5KU) 75 U/mL in the final oral bolus, pepsin (Sigma P7012, 2000 U/mL in the final gastric bolus), pancreatin (Sigma P7545, 100 TAME U/mL activity (one unit hydrolyses 1 μmol of p-toluene-sulfonyl-L-arginine methyl ester (TAME) per minute at 25 °C, pH 8.1) based on trypsin in the final intestinal bolus). Bile extract (Sigma B8631, 10 mM) was added to the intestinal bolus.

Test samples (black locust foliage/pod with simulated fluids, but without enzymes) were used to adjust the pH circumstances in each digestion phase.

Standards, as fatty acids, Folin-Ciocalteu reagent, gallic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), and Trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich Ltd. (St. Louis, USA) HPLC and analytical grade organic solvents (methanol, 1,2-dichloroethane,

acetonitrile, hexane, ethanol, acetone) and other chemicals (quartz sand, KH_2PO_4 , anhydrous Na_2SO_4) were purchased from VWR International (Radnor, USA). Reagents, standards, and eluents for amino acid analysis were purchased from Waters Corporation (Milford, USA). Acrylamide solution and molecular weight standard were bought from Bio-Rad (CA, USA). The inhibitor activity staining chymotrypsin was purchased from Fluka (Buchs, Switzerland), while the trypsin and the other reagents were from Sigma-Aldrich Ltd. (St. Louis, USA). The enzymes and bile extract used for simulated human in vitro gastrointestinal digestion were obtained from Sigma-Aldrich Ltd. (St. Louis, USA).

All other reagents were commercially available and of analytical grade.

Statistical analysis

All the samples were analyzed in triplicate. All data in the tables were presented as a mean number ($n=3$) and standard deviations. IBM SPSS Statistics.25 (Chicago, IL, USA, SPSS) software was used for normality analysis (Kolmogorov–Smirnov and Shapiro–Wilk tests), ANOVA (Tukey's test), principal component analysis (PCoA), and t-probe analysis.

Results

Genotyping the studied black locust cultivars and the putative polyploid clones

Eight of the 10 applied SSR loci produced a full fingerprint for all analyzed genotypes. In comparison, the amplification of two loci (Rops 02 and Rops 18) failed in five cases, resulting in putative null alleles. All the applied markers were polymorphic, and the main parameters can be found in Supplementary Table S1. The probability of identity (P_{ID}) was 4.032×10^{-11} , so the applied method proved reliable for fingerprinting individual genotypes. All the selected varieties have unique genotypes, while out of the added six polyploid selections, two were identical ('I1' and 'F1'), implying the same vegetative origin.

Regarding the allelic pattern of a possible polyploid state, only a two-allelic pattern was observed among all analyzed genotypes. A higher ploidy

level was not detectable based on a multi-allelic composition.

Based on the genetic relatedness derived from the SSR-based genetic distance matrix, the varieties ‘Kiskunsági’ (EL12) and ‘PL35’ (EL9) are clearly separated from the other genotypes, whereas the remaining samples form two main branches on the UPGMA dendrogram (Fig. 1). One branch comprises the cultivars ‘Debreceni-2’ (EL4), ‘Szajki’ (EL7), and ‘Üllői’ (EL1), together with the ‘I1’–‘F1’ clones, which were previously considered putative polyploids. The second branch includes the cultivars ‘PL324’ (EL5), ‘Váti’ (EL8), ‘PL251’ (EL6), ‘PL40’ (EL2), ‘Nyírségi’ (EL10), and ‘Zalai’ (EL3), along with the putative polyploid selections ‘K62’ and ‘4’.

Although ‘K62’ and ‘4’ are grouped within the same major cluster as ‘K68’ and ‘K66’, the genetic distance values do not indicate close genetic proximity among these genotypes. In contrast, ‘K68’ and ‘K66’ form a distinct pair, reflecting a higher degree of genetic similarity between them.

In this study, the term *cultivars* refers to the registered Hungarian black locust varieties included in the main analysis, whereas ‘K62’, ‘4’, ‘K66’, ‘K68’, ‘I1’,

and ‘F1’ are treated as putative polyploid selections of uncertain origin (see “Materials and methods” section). The principal component analysis (PCoA; Fig. 2), calculated from the same genetic distance matrix, provides an ordination of the genotypes that is consistent with the main clustering pattern observed in the UPGMA dendrogram, thereby offering a complementary visualization of the genetic relationships.

Main chemical composition of the foliage and pod samples of black locust cultivars

The dry matter, total protein, fat, and ash contents of the foliage and pod samples were determined, along with the total phenolic content and antioxidant capacity (Table 2). For comparison, a commercially available alfalfa pellet sample was also analyzed, as its chemical composition is similar to that of the black locust samples and it is widely used for feeding purposes (Ayers et al. 1996).

Significant differences were observed among the black locust foliage samples in dry matter content measured according to ISO 6496. The average dry matter content of the leaf samples was 93.8%. The

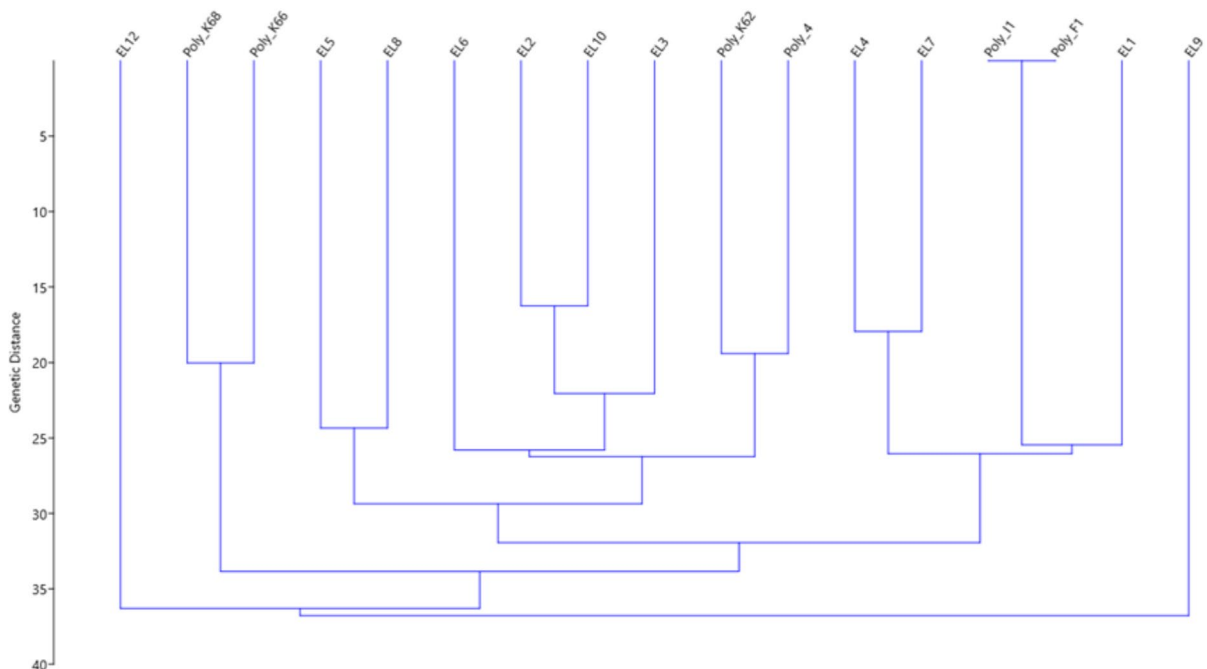


Fig. 1 UPGMA dendrogram based on multilocus SSR genetic distances showing relationships among the studied black locust genotypes

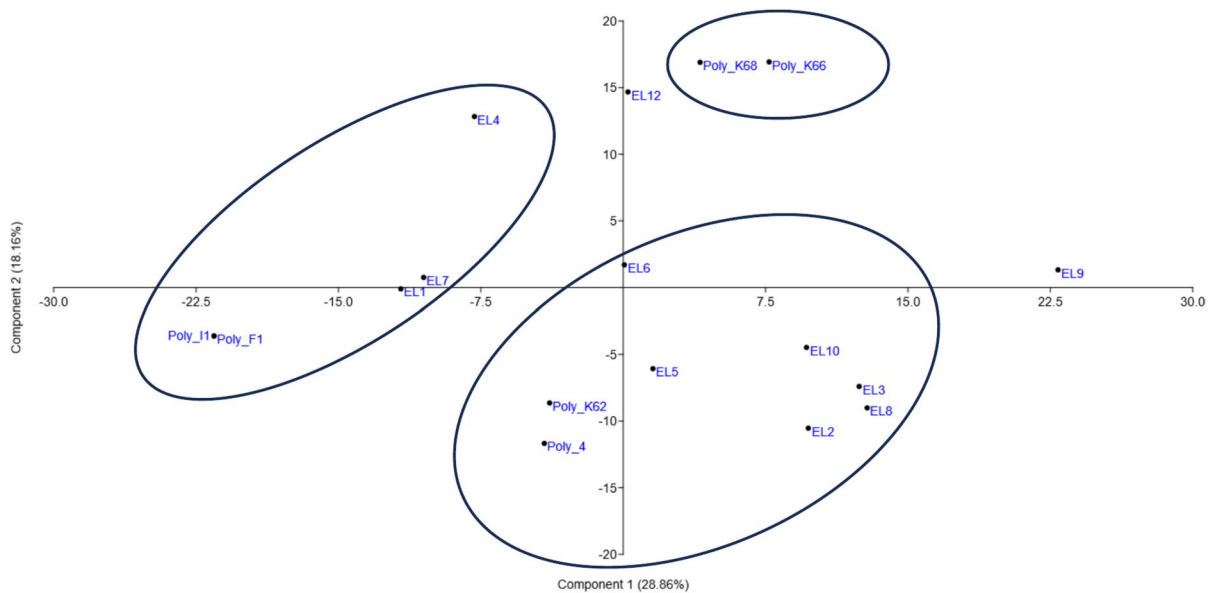


Fig. 2 Principal component analysis (PCoA) derived from the multilocus SSR genetic distance matrix. The first two axes explain 28.86% and 18.16% of the total variation, respectively

crude protein content of the foliage ranged between 15.5 and 20.0%, fat content between 4.1 and 6.0%, and ash content between 5.7 and 11.2%. When compared with alfalfa, the alfalfa pellet showed lower dry matter, crude protein, and fat contents (Table 2). In contrast, the ash content was significantly lower in black locust foliage than in alfalfa.

The average dry matter content of the black locust pod samples was 89.6%, which was lower than that of both the foliage and alfalfa samples. In addition, the protein, fat, and ash contents of the pods were also lower (16.4%, 3.2%, and 3.35%, respectively) compared to those of the foliage and alfalfa samples. The dry matter, total protein, fat, and ash content, as well as total phenolic content and antioxidant capacity, were measured from the foliage and pod samples (Table 2). For comparison, an alfalfa pellet sample was also examined, as its composition is very similar to black locust samples, and its use for feed purposes is widespread (Ayers et al. 1996).

From a nutritional and feeding perspective, the observed differences between black locust foliage and pod samples in both total polyphenol content and antioxidant capacity are particularly relevant. The markedly higher average total polyphenol content detected in the foliage (27,688 mg GAE/kg) compared with the pods (5970 mg GAE/kg) is consistent

with the substantially higher antioxidant capacity found in the foliage samples (198.2 vs. 24.3 mM Trolox/kg). This clear association suggests that phenolic compounds contribute decisively to the antioxidant potential of black locust leaves, highlighting their functional value beyond basic nutrient supply.

When compared with alfalfa, black locust foliage exhibited significantly higher total polyphenol content and antioxidant capacity (Table 2), indicating a potentially improved capacity to modulate oxidative processes when included in animal diets. Such properties may be particularly advantageous under intensive production conditions, where oxidative stress can negatively affect animal health and performance. In contrast, the total polyphenol content of black locust pods was comparable to that of alfalfa (5970 vs. 6170 mg GAE/kg); however, the significantly higher antioxidant capacity measured in the pods (24.3 vs. 9.6 mM Trolox/kg) suggests qualitative differences in the phenolic profile or improved bioactivity of the compounds present.

Amino acid content

The concentrations of individual amino acids in black locust foliage, pods, and alfalfa pellets are presented in Table 3. A comparison of the amino

Table 3 Amino acid composition of black locust foliage (EL), pods (ET), and Alfalfa pellet (% amino acid in protein)

	EL01		EL06		EL07		EL11		EL13		ET01		ET06		Alfalfa pellets	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Ser	5.78 b	0.12	5.85 b	0.15	6.06 b	0.09	5.84 b	0.07	5.92 b	0.06	7.02 a	0.19	6.72 a	0.13	6.03 b	0.11
Arg	5.78 c	0.11	5.6 c	0.2	5.57 c	0.09	5.64 c	0.09	5.64 c	0.07	8.87 b	0.11	9.7 a	0.12	5.4 c	0.09
Gly	5.93 b,c	0.25	5.5 c	0.19	5.33 c	0.12	5.94 b	0.09	5.88 b	0.08	6.53 a	0.09	5.94 b	0.07	4.4 d	0.1
Asp	8.76 c	0.25	12.46 b	0.19	14.18 a	0.31	8.81 c	0.15	8.78 c	0.11	8.74 c	0.12	8.82 c	0.13	14.39 a	0.25
Glu	9.55 b	0.22	9.27 b	0.24	8.7 c	0.19	9.45 b	0.26	9.24 b	0.31	13.63 a	0.45	14.26 a	0.34	9.92 b	0.26
Ala	6.9 a	0.31	6.81 a	0.19	6.48 b	0.15	6.9 a	0.14	6.89 a	0.09	5.21 c	0.2	5.16 c	0.23	6.83 a	0.15
Pro	6.47 b,c	0.31	7.08 b	0.42	6.58 b,c	0.19	6.62 b,c	0.32	6.47 b,c	0.28	6.19 c	0.19	6.04 c	0.35	9.44 a	0.41
Cys	1.22 a,b	0.18	0.99 b,c	0.2	1.08 a,b	0.25	1.2 a,b	0.31	1.44 a	0.19	1.2 a,b	0.24	0.74 c	0.09	1.32 a	0.19
Tyr	3.73 b,c	0.21	3.44 c	0.19	3.18 c	0.38	3.92 b	0.27	3.75 b,c	0.23	3.36 b,c	0.29	3.57 b,c	0.25	4.86 a	0.61
Thr	5.46 b	0.16	5.07 c	0.18	4.91 c	0.18	5.43 b	0.16	5.43 b	0.11	4.13 d	0.49	3.97 d	0.18	6.16 a	0.21
His	2.53 b	0.06	2.29 b	0.1	2.49 b	0.09	2.47 b	0.11	2.47 b	0.08	3.27 a	0.16	3.12 a	0.11	1.5 c	0.07
Lys	6.46 a,b	0.25	5.77 b	0.16	6.18 a,b	0.32	5.88 b	0.28	6.18 a,b	0.45	6.94 a	0.35	6.62 a	0.29	5.95 b	0.26
Met	1.39 a,b	0.26	1.14 b	0.15	1.08 b	0.19	1.38 a,b	0.18	1.52 a	0.12	0.91 c	0.2	0.9 c	0.19	1.24 b	0.05
Val	6.94 a	0.26	6.83 a	0.5	6.65 a	0.38	6.94 a	0.31	6.97 a	0.27	5.65 b	0.41	5.66 b	0.37	0.75 c	0.07
Ile	5.84 a	0.27	5.61 a	0.36	5.45 a	0.24	5.88 a	0.41	5.89 a	0.39	4.63 b	0.29	4.68 b	0.32	5.9 a	0.29
Leu	10.48 a	0.67	9.8 a	0.58	9.47 a	0.49	10.6 a	0.67	10.45 a	1.05	8.23 b	0.57	8.43 b	0.59	9.63 a	0.68
Phe	5.98 a	0.61	5.74 a	0.45	5.84 a	0.48	6.17 a	0.39	6.11 a	0.29	4.42 b	0.31	4.57 b	0.28	6.01 a	0.51
Trp	0.8 b,c	0.06	0.73 c	0.07	0.77 b,c	0.06	0.93 b	0.11	0.99 a,b	0.09	1.12 a	0.06	1.11 a,b	0.12	0.26 d	0.02

Different letters indicate statistically significant differences within rows according to Tukey's multiple range test ($p < 0.05$)

acid profiles of foliage and pod samples revealed marked qualitative and quantitative differences, indicating tissue-specific protein composition. The concentrations of several amino acids, including Asp, Thr, Ala, Met, Val, Ile, Leu, and Phe, were significantly lower in the pods than in the foliage. In contrast, His, Ser, Gly, Arg, Lys, Trp, and Glu were present at significantly higher levels in the pod samples, suggesting a distinct role of black locust reproductive organs in the allocation of nitrogen and amino acids.

Compared with alfalfa, the amino acid composition of black locust foliage exhibited both advantages and limitations. Alfalfa contained significantly higher amounts of Asp, Thr, Pro, and Tyr, whereas Gly and Trp concentrations were lower than those detected in black locust leaves. In the case of black locust pods, comparison with alfalfa revealed significantly higher concentrations of His, Ser, Arg, Gly, Lys, Glu, Val, and Trp. Conversely, the pods contained significantly lower levels of Asp, Thr, Ala, Pro, Cys, Tyr, Met, Ile, Leu, and Phe. These differences highlight that, despite a generally lower protein concentration, black

locust pods may contribute valuable specific amino acids to animal diets.

Among the foliage samples, EL06 and EL07 exhibited significantly higher Asp concentrations compared to the other leaf samples. Notably, these two samples were also characterized by higher crude protein contents, despite originating from the same location and being collected at the same sampling date as the remaining samples (Table 3). This observation suggests that factors beyond sampling site and time, such as individual tree physiology or micro-environmental conditions, may influence amino acid accumulation in black locust foliage.

From a nutritional standpoint, the comparison of amino acid profiles across alfalfa, black locust leaves, and pods revealed that the essential amino acids His, Val, and Trp were present at significantly higher levels in both black locust tissues than in alfalfa. Among the essential amino acids, only Thr showed significantly higher concentrations in alfalfa compared with the black locust samples. Given the importance of His, Val, and Trp in protein synthesis, metabolic regulation, and immune function, these findings further

support the potential use of black locust foliage—and to a lesser extent pods—as alternative or complementary protein sources in animal nutrition.

Fatty acid content

The fatty acid composition further revealed pronounced differences between black locust foliage and pod samples (Table 4), which may have important nutritional implications. The proportion of saturated fatty acids was significantly higher in the foliage samples, whereas the pods contained significantly higher amounts of monounsaturated and polyunsaturated fatty acids. Black locust foliage was characterized mainly by the presence of α -linolenic acid (C18:3n3), linoleic acid (C18:2n6c), and several saturated fatty acids, including lauric, myristic, palmitic, stearic, and heneicosanoic acids.

In contrast, the fatty acid profile of the pods was dominated by unsaturated fatty acids. Monounsaturated oleic acid (C18:1n9), together with the polyunsaturated α -linolenic and linoleic acids, accounted for more than 85% of the identified fatty acids in the pod samples, whereas their combined proportion did not exceed 60% in the foliage. This shift toward a higher degree of unsaturation suggests distinct metabolic roles of vegetative and reproductive tissues and increases the nutritional relevance of pod lipids. Notably, myristic acid and arachidonic acid were detected exclusively in the foliage samples, indicating tissue-specific fatty acid synthesis or accumulation.

From a feeding perspective, the fatty acid composition of both black locust leaves and pods appears particularly favorable when compared with alfalfa pellets. In both plant tissues, the proportion of unsaturated fatty acids was significantly higher than that observed in alfalfa, which may contribute to improved lipid quality in animal diets. Given the recognized benefits of polyunsaturated fatty acids—especially n-3 fatty acids such as α -linolenic acid—in modulating inflammatory responses, metabolic health, and product quality, black locust foliage and pods may represent valuable alternative lipid sources in forage-based feeding systems.

Determination of phenolic components

Based on the results of the total polyphenol analyses, the total polyphenol content of black locust leaves

was approximately one order of magnitude higher than that measured in the pods (Table 2). Consequently, detailed polyphenol profile analyses were performed exclusively on leaf samples.

Flavonoid compounds and their derivatives with molecular weights up to 1000 Da were analyzed. Compounds with higher molecular weights were not included in the analysis due to the maximum sensitivity limit of the QDA detector (1000 Da). As a result, discrepancies can be observed between the values obtained for total phenolic content and the compound-specific quantitative data. Acacetin and its various glycoside derivatives were detected at the highest concentrations and together accounted for approximately 90% of the identified phenolic compounds (Table 5). In addition, glycosides and derivatives of luteolin, apigenin, and diosmetin were also identified, although in substantially lower concentrations.

According to Table 6, the sum of the quantified phenolic compounds, expressed as apigenin equivalents, ranged from 1220 to 1700 mg/100 g dry matter. Detailed analysis revealed that, in all analyzed samples, acacetin conjugated with deoxyhexose and hexose was the dominant phenolic constituent. The concentration of these acacetin conjugates varied between 415 and 786 mg/100 g defatted sample, with the lowest and highest values observed in samples EL 11 and EL 13, respectively.

Fermentation of black locust by lactic acid bacteria to enhance the digestibility

The living *Lactobacillus* cell count reached 10^8 cfu/mL ($1.51\text{--}7.05 \times 10^8$ cfu/mL) from the initial $6.83 \times 10^6\text{--}3.5 \times 10^7$ cells/mL concentration, except in sample EL11 (Fig. 3). The original pH of the black locust samples was around 6 (5.8–6.16) and decreased to 4.85–5.29 due to the acid production of *Lactobacillus*. The black locust samples are fermentable; however, the extent of fermentation is limited because the pH has not decreased below 4, and the cell growth has only one degree of freedom.

The protein composition of the samples was analyzed by 6/15% SDS-PAGE for the initial, fermented, and digested black locust foliage and pod samples (Fig. 4).

Concerning the different cultivation areas, we found no differences in the protein distribution of the initial samples. Black locust foliage contained

Table 4 Fatty acid composition (%) of black locust leaves (EL), pods (ET), and alfalfa pellet

	EL01		EL06		EL07		EL11		EL13		ET01		ET06		Alfalfa pellet	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C14:0	1.93 d	0.03	4.81 a	0.2	3.41 b	0.01	2.82 c	0.02	1.64 e	0.04	nd	nd	nd	nd	0.55 f	0.05
C16:0	14.43 b	0.21	16.84 a	0.46	13.23 b	0.26	16.74 a	0.19	14.25 b	0.12	5.43 c	0.08	5.61 c	0.05	13.96 b	1.18
C18:0	2.04 d	0.03	2.72 b	0.09	2.75 b	0.02	3.39 a	0.27	2.72 b	0.01	2.64 c	0.02	2.8 b	0.03	2.82 b	0.23
C18:1n9c	2.83 e	0.02	2.75 e,f	0.06	2.69 f	0.02	6.19 c	0.07	2.58 f	0.02	11.38 b	0.09	12.29 a	0.13	3.50d	0.30
C18:2n6c	7.13 f	0.09	6.97 f	0.31	8.19 e	0.07	10.95 c	0.19	9.56 d	0.03	58.82 a	0.4	58.66 a	0.81	12.46 b	1.06
C18:3n3	45.21 a	0.48	22.49 e	0.68	26.14 d	0.19	35.69 c	0.54	41.38 b	1.26	16.02 f	0.06	13.5 g	0.45	13.90 g	1.13
C21:0	2.48 c	0.02	5.77 a	0.62	6.34 a	0.01	6.11 a	0.04	3.85 b	0.93	1.71 d	0.21	0.94 e	0.02	nd	nd
C20:4n6	1.7 c	0.02	3.01 a	0.28	3.29 a	0.05	2.64 b	0.05	1.58 c	0.48	nd	nd	nd	nd	3.11 a	0.33
Total	77.75 c	1.03	65.36 d	0.93	66.04 d	0.33	84.53 b	1.22	77.56 c	0.92	96 a	0.38	93.8 a	0.68	50.03 e	0.68
Saturated	20.88 c	0.26	30.14 a	0.06	25.73 b	0.01	29.06 a	0.37	22.46 c	1.18	9.78 e	0.05	9.35 f	0.1	17.33 d	0.10
Monounsaturated	2.83 e	0.17	2.75 e	0.06	2.69 e	0.02	6.19 c	0.07	2.58 e	0.54	11.38 b	0.09	12.29 a	0.13	3.50 d	0.13
Polyunsaturated	54.04 c	0.59	32.47 g	0.93	37.62 f	0.33	49.28 e	0.78	52.52 d	0.8	74.84 a	0.34	72.16 b	0.65	29.47 h	0.65

nd not detectable, the concentration is below the detection limit

Different letters indicate statistically significant differences within rows according to Tukey's multiple range test ($p < 0.05$)

Table 5 The most important phenolic compounds found in the black locust foliage samples

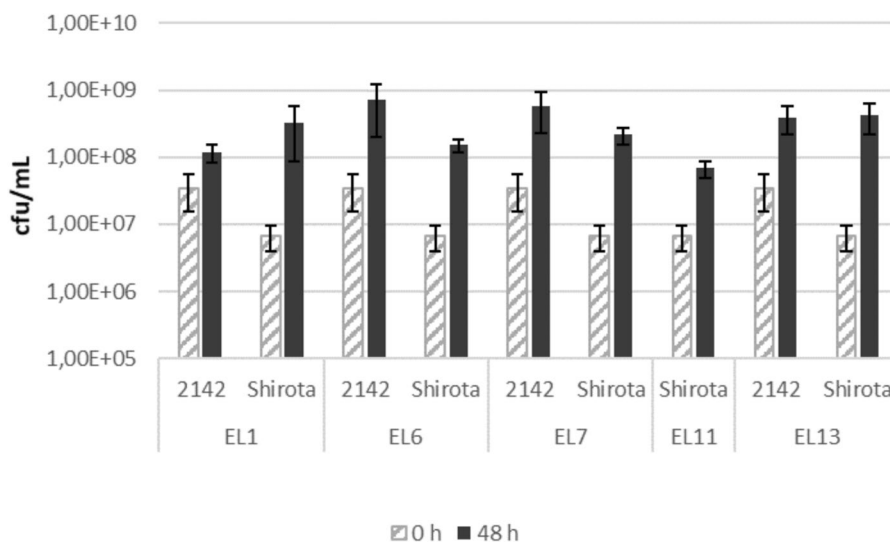
Peak no.	Retention time (min)	UV _{max} (nm)	[M + H] ⁺ m/z ions	[M-H] ⁻ m/z ions	Aglycon	Glycosidic components		
1.	7.46	254, 266sh, 349	595	499, 287	593	285	Luteolin	Deoxyhexose, hexose
2.	7.91	254, 267sh, 348	449	287	447	285	Luteolin	Hexose
3.	8.22	267 334	579	433, 271	577	269	Apigenin	Deoxyhexose, hexose
4.	8.70	253, 267sh, 346	609	463, 301	607	299, 284	Diosmetin	Deoxyhexose, hexose
5.	8.79	267, 332	769	623, 447, 285	767	283, 268	Acacetin	Deoxyhexose, hexose, galacturonic acid
6.	9.14	267, 333	755	609, 447, 285	753	283, 268	Acacetin	Deoxyhexose, hexose, hexose
7.	9.68	267, 333	725	579, 447, 285	723	283, 268	Acacetin	Deoxyhexose, hexose, pentose
8.	10.43	267, 332	593	447, 285	591	283, 268	Acacetin	Deoxyhexose, hexose

Table 6 Phenolic glycoside compounds in black locust foliage (EL) samples

	Luteolin-deoxyhexose-hexose	Luteolin-hexose	Apigenin-deoxyhexose-hexose	Diosmetin-deoxyhexose-hexose	Acacetin-deoxyhexose-hexose-galacturonic acid	Acacetin-deoxyhexose-dihexose;	Acacetin-deoxyhexose-hexose-pentose	Acacetin-deoxyhexose-hexose
	mg/100 g Apigenin equivalent (mean ± SD)							
EL01	19.2 ± 0.12 d	33.86 ± 1.53 b	13.22 ± 0.14 f	5.64 ± 0.1 d	360.75 ± 9.23 a	133.34 ± 3.99 d	310.32 ± 6.72 c	532.82 ± 18.22 c
EL06	47.88 ± 0.38 b	36.5 ± 1.06 b	29.71 ± 0.62 b	6.93 ± 0.15 c	356.43 ± 8.46 a	208.52 ± 4.95 a	406.23 ± 9.76 a	607.22 ± 13.76 b
EL07	34.78 ± 1.13 c	17.96 ± 0.13 d	22.38 ± 1.94 c,d	65.91 ± 2.01 a	317.68 ± 22.49 b	111.67 ± 6.14 e	326.28 ± 19.11 c,b	611.26 ± 41.42 b
EL09	64.56 ± 3.46 a	34.13 ± 2.01 b	25.82 ± 1.39 c	16.99 ± 1.29 b	114.52 ± 10.14 e	146.31 ± 11.97 c	363.17 ± 30.33 b	643.22 ± 50.2 b
EL10	68.97 ± 0.39 a	40.81 ± 0.59 a	21.19 ± 0.56 d	67.62 ± 1.98 a	329.49 ± 9.93 b	113.94 ± 3.81 e	312.21 ± 9.77 c	594.31 ± 18.73 b
EL11	10.44 ± 0.46 e	24.09 ± 0.32 c	32.14 ± 0.15 a	7.08 ± 0.02 c	250.22 ± 2.79 c	150.24 ± 1.78 c	295.98 ± 3.39 c	785.79 ± 5.57 a
EL13	10.65 ± 0.03 e	18.79 ± 0.32 d	16.55 ± 0.03 e	6.55 ± 0.41 c	225.95 ± 3.69 d	166.12 ± 2.76 b	357.02 ± 4.35 b	415.89 ± 2.4 d

Luteolin-deoxyhexose-hexose; luteolin-hexose; apigenin-deoxyhexose-hexose; diosmetin-deoxyhexose-hexose; acacetin-deoxyhexose-hexose-galacturonic acid; acacetin-deoxyhexose-dihexose; acacetin-deoxyhexose-hexose-pentose; acacetin-deoxyhexose-hexose

Different letters indicate statistically significant differences within columns according to Tukey's multiple range test ($p < 0.05$)

Fig. 3 The *Lactobacillus* cell count, initial and after 48 h of fermentation in black locust samples

few protein bands, with the most intense protein bands observed in the range of 14 and 60 kDa, while black locust pods contained many more protein bands than black locust foliage, in the molecular weight range of 5–250 kDa (Fig. 4/samples 2–4).

After black locust foliage samples were fermented by *Lb. casei*, Shirota, and *Lb. plantarum* 2142, the protein bands decreased in intensity and faded, especially in the 14 and 60 kDa bands, but no complete degradation of the proteins occurred with the applied microbial strains (Fig. 4/samples 12–17).

In contrast to fermentation, our examination of the small intestinal phase of in vitro digestion revealed that proteins were extensively degraded in foliage and pods. The number of proteins was significantly reduced and absent in the upper, high-molecular-weight bands. In the lower molecular weight range, more peptides/proteins are visible (existing or newly formed). In several cases, a diffuse zone also appeared, referring to hydrolysis. The fact mentioned above is more typical for foliage, while the absence of a diffuse zone in the pod indicates a more complete degradation of the proteins (Fig. 4/samples 6–8).

However, protein bands are visible in the samples, primarily in the higher-molecular-weight range. These are the protein fractions of the digestive enzymes that remained. Their presence is certified by alpha-amylase, pepsin, and pancreatin as controls. (Fig. 4/samples 10–11).

Regarding the digestion of fermented foliage, fermentation helped isolate the protein assays from other confounding factors. A more apparent background was obtained on the electrophoretic image, but the fermentation itself did not cause any further significant improvement in the digestion results (Fig. 4/samples 18–21).

Native PAGE is a proven and appropriate method for identifying the antinutritive trypsin inhibitors that may be present in foliage and pods. Following protein separation, inhibition of Kunitz-type trypsin, Bowman–Birk-type trypsin, and chymotrypsin was detected by enzyme staining (trypsin, TRY; chymotrypsin, CHY). Under native conditions, the foliage proteins accumulated at the top of the gel due to their high molecular weights. They concentrated in 3–4 main bands in the pod samples (Fig. 5/a). Proteins similar to KTI and BBI activity were detected only

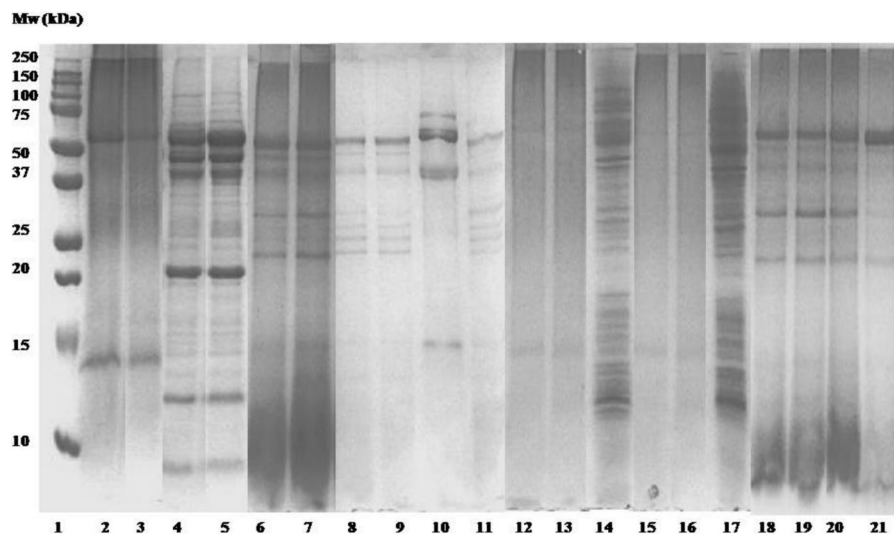


Fig. 4 Monitoring the effect of digestion and fermentation on the protein separation of black locust foliage (EL) and pods (ET) by 6/15% SDS-PAGE technique (1—molecular weight standard, 2—EL1, 3—EL6, 4—ET1, 5—ET6, 6—EL1—intestinal digested phase, 7—EL6—intestinal digested phase, 8—ET1—intestinal digested phase, 9—ET6—intestinal digested phase, 10—human alpha-amylase (58 and 62 kDa, Sigma A1031)+porcine pepsin (35 kDa, Sigma P7012), 11—porcine pancreatin (Sigma P7545), 12—EL1—fermented by *Lb. casei*

Shirota, 13—EL6—fermented by *Lb. casei* Shirota, 14—*Lb. casei* Shirota, —EL1—fermented by *Lb. plantarum* 2142, 16—EL6—fermented by *Lb. plantarum* 2142, 17—*Lb. plantarum* 2142, 18—EL1—fermented by *Lb. casei* Shirota, then digested (intestinal phase), 19—EL6—fermented by *Lb. casei* Shirota, then digested (intestinal phase), 20—EL1—fermented by *Lb. plantarum* 2142, then digested (intestinal phase), 21—EL6—fermented by *Lb. plantarum* 2142, then digested (intestinal phase)

in pod samples; however, they differed from those of soybean KTI and BBI at band height (Fig. 5/b, c).

Discussion

According to the relevant international literature, the raw protein content of black locust (*Robinia pseudo-acacia*) is close to that of alfalfa (*Medicago sativa*). Black locust is an excellent fodder plant for pig fattening and poultry production (Papachristou 1999; Ayers et al. 1996), and its foliage has also been reported to promote antler development in deer (Halász et al. 2021). In contrast to most conventional protein crops, black locust can be cultivated under extreme site conditions, making it a potentially resilient alternative forage resource in increasingly arid climates.

In the present study, foliage and pod samples were collected from two experimental plantations located at distinct sites in Püspökladány and Hajdúhadház (Hungary). Eleven black locust cultivars were evaluated for foliage composition, including two clones present at both experimental sites, while pod samples were obtained from ten cultivars at one location. Based on the comprehensive chemical and biochemical analyses, several preliminary conclusions can be drawn regarding the nutritional potential of black locust biomass.

From a protein nutrition perspective, black locust foliage exhibited crude protein levels comparable to alfalfa and a favorable amino acid profile. The relatively high concentrations of essential amino acids such as histidine, valine, and tryptophan observed in both foliage and pods suggest that black locust biomass may help improve amino acid balance in feeding. Although alfalfa contained significantly higher levels of lysine and threonine, which are known to be limiting in grain-based feeding systems (Denis et al. 2023), the methionine content of black locust leaves reached values comparable to those reported for alfalfa, in agreement with earlier literature (Ma et al. 2022). These findings indicate that black locust foliage, similar to alfalfa, may be suitable for dietary supplementation to achieve a balanced amino acid supply.

The lipid fraction of black locust tissues was characterized by a relatively high proportion of unsaturated fatty acids, with clear differences between vegetative and reproductive organs. Both foliage and pods contained higher total fatty acid levels and higher proportions of mono- and polyunsaturated fatty acids compared with alfalfa, supporting previous observations regarding forage lipid composition (Vlaicu et al. 2023). Given the recognized role of linoleic and α -linolenic acids in ruminant health and reproduction (Denis et al. 2023), these characteristics may be

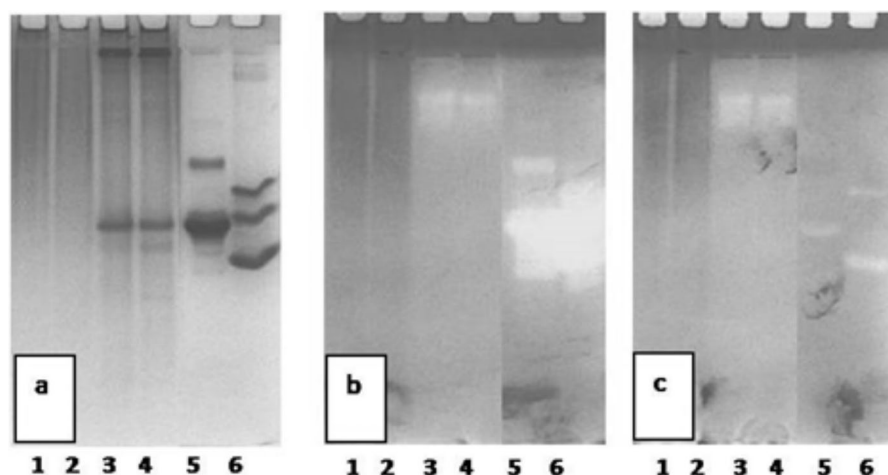


Fig. 5 Searching the possible trypsin inhibitor type antinutrient proteins of black locust foliage (EL) and pods (ET) by 6/15% native PAGE separation (a); stained 6/15% native PAGE separation after treated with trypsin (b); stained 6/15% native PAGE separation after treated with chymotrypsin (c). KTI:

Kunitz type trypsin inhibitor from *Glycine max* (soybean); BBI: Bowman–Birk type trypsin–chymotrypsin inhibitor from *Glycine max* (soybean); 1: EL1; 2: EL6; 3: ET1; 4: ET6; 5: KTI (Sigma T2327; 6: BBI (SIGMA T 9777)

nutritionally relevant. However, their practical importance will depend on factors such as intake, digestibility, and animal species.

One of the most pronounced differences between black locust and alfalfa was observed in total polyphenol content and antioxidant capacity. Black locust foliage contained approximately one order of magnitude higher total polyphenol levels than pods and significantly higher values than alfalfa, consistent with published data on legume forages (Guo et al. 2019; Horvat et al. 2022; Raeeszadeh et al. 2022). Detailed phenolic profiling revealed a conserved flavonoid pattern dominated by acacetin and its glycoside derivatives, which have been previously identified as major phenolic constituents of *R. pseudoacacia* (Tian and McLaughlin 2000). The predominance of acacetin derivatives may partly explain the high antioxidant capacity of black locust foliage and supports earlier reports on the biological relevance of these compounds (Semwal et al. 2019; Lukash et al. 2024).

At the same time, the presence of antinutritional factors, particularly tannins and protease inhibitors, represents a relevant limitation to the direct use of black locust biomass in animal feeding. Earlier studies demonstrated reduced digestibility and growth performance when black locust foliage was included at high dietary levels, mainly due to tannin-related effects (Ayers et al. 1996; Rakesh et al. 2000). The results of the present study indicate that microbial processing, especially lactic acid fermentation, can partially mitigate these limitations by enhancing protein degradation and potentially improving digestibility. This is consistent with previous observations of tannin depolymerization by *Lactobacillus* strains (Rodríguez et al. 2008) and supports fermentation as a promising processing strategy.

Several strategies have been proposed to reduce tannin content and improve the digestibility of tannin-rich forages. Rakesh et al. (2000) demonstrated the effectiveness of *Aspergillus niger* treatment in lowering tannin levels in black locust foliage, while Wina et al. (2008) reported successful tannin reduction in *Calliandra calothyrsus* using polyethylene glycol (PEG) and calcium hydroxide. In addition, Rodríguez et al. (2008) showed that *Lactobacillus plantarum* is capable of degrading gallotannins through the depolymerization of high-molecular-weight tannins and converting low-molecular-weight tannins into gallic acid and pyrogallol. In

the present study, we aimed to extend these findings by examining whether lactic acid fermentation could also contribute to the degradation of other antinutritional components, such as protease inhibitors, in addition to tannins.

Protease inhibitors are known to exert both antinutritional and health-related effects depending on their concentration and structural characteristics. Antinutritive protease inhibitors can inhibit digestive enzymes, including trypsin and chymotrypsin, through competitive binding, thereby reducing protein digestibility and animal growth. Consequently, the occurrence of such proteins should be evaluated prior to the inclusion of foliage as a feed component. The protein profiles of black locust leaves were relatively uniform, characterized by a limited number of protein bands, with the most intense signals observed between 14 and 60 kDa. No differences in protein distribution were detected between samples originating from the two experimental sites (clone ‘Üllői’: EL1 and EL11; clone ‘Szajki’: EL7 and EL13).

In contrast, black locust pods exhibited more protein bands than leaves. Comparisons among pod samples revealed differences in band intensity but not in overall protein composition. Pod samples ET5 (clone ‘PL324’) and ET7 (clone ‘Szajki’), originating from the same experimental site, showed notably weaker band intensity. To assess the potential presence of protease inhibitors contributing to the higher protein content observed in pod samples, stained native PAGE analysis was performed. The results indicated the presence of inhibitor activity in pod samples; however, the activity pattern differed from that reported for soybean (*Glycine max*, Kollipara and Hymowitz 1992). Further studies are required to characterize these inhibitors and to evaluate their relevance in the context of animal feeding.

Both the fermentation experiments using *Lactobacillus* strains and the in vitro digestion assays demonstrated enhanced protein breakdown following treatment, suggesting improved digestibility of black locust leaves. Given that tannins—the other major antinutritional components of black locust—are also susceptible to depolymerization by *Lactobacillus* strains (Rodríguez et al. 2008), the inclusion of lactic acid fermentation appears to be a promising approach when black locust biomass is intended for feed use.

A joint genetic analysis was also included in the study to check the ploidy level of black locust

genotypes. A first attempt to select polyploid black locust trees from various sources for further feedstock breeding was initiated in Hungary in the 1960s (Keresztesi 1984). Cytological examinations identified several mixoploid and tetraploid individuals, and the chimera phase could be proved in the case of mixoploids. The selected polyploid genotypes showed unique leaf morphological properties: much larger leaf area compared to the average black locust foliage due to the increased size and thickness of the leaf blade. Unfortunately, this breeding direction persisted for a few years, leaving behind only a few selected genotypes in a gene collection, and the focus of breeding interest shifted again toward wood production. Genetic fingerprinting using SSR (simple sequence repeat) DNA markers is a useful starting point for assessing putative polyploid genotypes based on their multi-allele patterns (Weising et al. 2005). The genetic analysis carried out in the present study does not support a higher ploidy level among the analyzed genotypes. The applied fingerprinting technique showed no aberration from the diploid allelic pattern. The six additional putative polyploid clones analyzed showed allelic patterns congruent with those of the other samples. Therefore, the question of higher yield connected to higher ploidy level remained unanswered, and further analyses are needed (cytological evaluation, leaf morphology assessments, yield measurements, etc.).

The harvesting of black locust pods in this study occurred at an unconventional, transitional phenological stage: the pods were undergoing senescence, while the seeds were almost ripe but not yet fully mature. From a feed science perspective, analyzing the whole fruit (pod and seed combined) at this stage offers distinct advantages and methodological insights highly relevant to future animal nutrition research.

This transitional phase represents a critical chemical turning point. While young, green pods are naturally more palatable to grazing ruminants due to higher moisture and lower fiber content, their seed protein yield is minimal. Conversely, fully mature pods have heavily lignified, unpalatable walls and stone-hard seed coats that require substantial milling energy. The intermediate stage analyzed here captures the point where seeds have already accumulated substantial protein (often ranging between 30 and 40% in crude protein), yet the seed coat has not fully

hardened, potentially easing mechanical processing. However, black locust is known to contain anti-nutritional factors and toxins, such as lectins and toxalbumins (e.g., robin). The onset of pod senescence likely triggers a major redistribution of these secondary metabolites between the pod wall and the developing seed, which directly affects the material's safety and palatability (Halász et al. 2021).

Analyzing the whole fruit at this stage is highly practical for livestock applications. In realistic agricultural scenarios, black locust pods are harvested and processed collectively as a whole-pod meal rather than separating the seeds. Therefore, our data provides a realistic baseline for the raw nutritional value an animal would encounter before any technological intervention.

To build upon these findings and optimize black locust as an alternative protein source, future research should consider the following approaches:

Dynamic sampling and tissue-specific analysis:

Future trials should evaluate the biomass across three distinct phases (early green, transitional, and fully mature) while analyzing the pod walls and seeds both separately and combined. This would map the exact migration of toxins and nutrients during maturity, identifying the safest harvest window.

Evaluating processing technologies: Since the evaluated stage still carries risks of toxicity and high fiber, future work must focus on processing treatments. Testing the effects of thermal treatments (such as roasting or extrusion) to denature heat-sensitive toxalbumins (Makkar and Becker 1999), or solid-state fermentation (silage) to break down anti-nutrients, will be crucial to unlocking the full feed potential of this biomass (de Barros et al. 2024).

Overall, the findings suggest that black locust foliage—and, to a lesser extent, pods—possess a combination of protein, lipid, and phenolic characteristics that may complement conventional forages such as alfalfa, particularly under conditions requiring resilience to environmental stress. While the main compositional traits were relatively stable across cultivars and sites, further studies focusing on digestibility, bioavailability, animal performance, and optimized processing methods are required to fully assess the

practical applicability of black locust biomass in animal feeding systems.

Acknowledgements This study was completed within the TKP2020-NKA-24 project framework, which has been implemented with the support provided by the Ministry of Innovation and Technology of Hungary (successor: Ministry of Culture and Innovation of Hungary) from the National Research, Development and Innovation Fund, financed under the TKP funding scheme.

Author contributions All authors contributed to the study's conception and design. Material preparation, data collection and analysis were performed by Rita Tömösközi-Farkas, Éva Lengyel-Kónya, Mária Berki, Zsolt Zalán, Krisztina Takács, Emőke Szerdahelyi, Enikő Szanics, Nóra Adányi, Attila Borovics, János Rásó, Klára Cseke, Tamás Molnár, Zsolt Keszérű. The first draft of the manuscript was written by Zsolt Keszérű, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding Open access funding provided by Hungarian University of Agriculture and Life Sciences. This study was made in the frame of the project TKP2020-NKA-24, implemented with the support provided by the Ministry of Innovation and Technology of Hungary (successor: Ministry of Culture and Innovation of Hungary) from the National Research, Development and Innovation Fund, financed under the TKP funding scheme.

Data availability All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethical approval Not applicable.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Ábri T, Cseke K, Keszérű Z, Porcsin A, Szabó FM, Rédei K (2023) Breeding and improvement of black locust (*Robinia pseudoacacia* L.) with a special focus on Hungary: a review. *iForest* 16:290–298. <https://doi.org/10.3832/for4254-016>
- AOAC Official Method 968.06 (2003) Protein (crude) in animal feed. Dumas method
- Ayers AC, Barrett RP, Cheeke PR (1996) Feeding value of tree leaves (hybrid poplar and black locust) evaluated with sheep, goats and rabbits. *Anim Feed Sci Technol* 57(1–2):51–62
- Blume L, Hoischen-Taubner S, Sundrum A (2021) Alfalfa—a regional protein source for all farm animals. *Landbau-forschung Appl Agric for Res* 71:1–13. <https://doi.org/10.3220/LBF1615894157000>
- Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. *LWT Food Sci Technol* 28:25–30
- Browne DT, Aguilo-Seara G, DeFranzo A Jr (2020) The black locust tree: toxalbumin-induced tissue necrosis of the upper extremity. *Cureus*. <https://doi.org/10.7759/cureus.11758>
- Burner DM, Carrier DJ, Belesky DP, Pote DH, Ares A, Clausen EC (2007) Yield components and nutritive value of *Robinia pseudoacacia* and *Albizia julibrissin* in Arkansas, USA. *Agrofor Syst* 72(1):51–62. <https://doi.org/10.1007/s10457-007-9098-x>
- Carl C, Landgraf D, van der Maaten-Theunissen M, Biber P, Pretzsch H (2017) *Robinia pseudoacacia* L. flower analyzed by using an unmanned aerial vehicle (UAV). *Remote Sens* 9:1091. <https://doi.org/10.3390/rs9111091>
- Chen Y, Zhao Y, Fu ZY, Ma ZW, Qian FC, Aibibuli A, Yang B, Abula R, Xu XL, Aniwaer A (2011) Chemical composition and in vitro ruminal fermentation characteristics of tetraploid black locust (*Robinia pseudoacacia* L.). *Asian J Anim Vet Adv* 6(7):706–714
- Comai L (2005) The advantages and disadvantages of being polyploid. *Nat Rev Genet* 6:836–846. <https://doi.org/10.1038/nrg1711>
- Corneillie S, De Storme N, Van Acker R, Fangel JU, De Bruyne M, De Rycke R, Geelen D, Willats WGT, Vanholme B, Boerjan W (2019) Polyploidy affects plant growth and alters cell wall composition. *Plant Physiol* 179:74–87. <https://doi.org/10.1104/pp.18.00967>
- de Barros CR, Ferreira LMM, Fraga I, Mourão JL, Rodrigues MAM (2024) Detoxification methods of *Jatropha curcas* seed cake and its potential utilization as animal feed. *Fermentation* 10:256. <https://doi.org/10.3390/fermentation10050256>
- Denis P, Schmidely P, Nozière P et al (2023) Predicted essential fatty acid intakes for a group of dairy cows also apply at individual animal level. *Animal* 17(11):101005. <https://doi.org/10.1016/j.animal.2023.101005>
- Dumolin S, Demesure B, Petit RJ (1995) Inheritance of chloroplast and mitochondrial genomes in pedunculate oak investigated with an efficient PCR method. *Theor Appl Genet* 91:1253–1256. <https://doi.org/10.1007/BF00220937>

- Farkas A, Zajác E (2007) Nectar production for the Hungarian honey industry. *Eur J Plant Sci Biotechnol* 1(2):125–151
- Giovanetti M, Ramos M, Máguas C (2018) Why so many flowers? A preliminary assessment of mixed pollination strategy enhancing sexual reproduction of the invasive *Acacia longifolia* in Portugal. *Web Ecol* 18(1):47–54. <https://doi.org/10.5194/we-18-47-2018>
- Guo H, Wan X, Niu F et al (2019) Evaluation of antiviral effect and toxicity of total flavonoids extracted from *Robinia pseudoacacia* cv. Idaho. *Biomed Pharmacother* 118:109335. <https://doi.org/10.1016/j.biopha.2019.109335>
- Halász A, Bajnok M, Suli A, Jonas Mikone E (2021) Importance of black locust (*Robinia pseudoacacia*) foliage in the extension of the grazing season and in the reduction of damages caused by climate change (a review). *J Rangel Sci* 11:119–124
- Hammer Ø, Harper DAT, Ryan PD (2001) PAST: paleontological statistics software package for education and data analysis. *Palaeont Electr* 4:9
- Horvat D, Viljevac Vuletić M, Andrić L, Baličević R, Kovačević Babić M, Tucak M (2022) Characterization of forage quality, phenolic profiles, and antioxidant activity in alfalfa (*Medicago sativa* L.). *Plants Basel* 11(20):2735. <https://doi.org/10.3390/plants11202735>
- Hsu YL, Kuo PL, Lin CC (2004) Acacetin inhibits the proliferation of Hep G2 by blocking cell cycle progression and inducing apoptosis. *Biochem Pharmacol* 67(5):823–829. <https://doi.org/10.1016/j.bcp.2003.09.042>
- Hui A, Marraffa JM, Stork CM (2024) A rare ingestion of black locust tree. *J Toxicol Clin Toxicol* 42(1):93–95. <https://doi.org/10.1081/CLT-120028752>
- ISO 6496:1999 (1999) Animal feeding stuffs—determination of moisture and other volatile matter content
- ISO 6492:1999 (1999) Animal feeding stuffs—determination of fat content
- ISO 2171:2023 (2023) Cereals, pulses and by-products—determination of ash yield by incineration
- Keresztesi B (1983) Breeding and cultivation of black locust (*Robinia pseudoacacia* L.) in Hungary. *For Ecol Manag* 6:217–244. [https://doi.org/10.1016/S0378-1127\(83\)80004-8](https://doi.org/10.1016/S0378-1127(83)80004-8)
- Keresztesi B (1984) Az akác nemesítése. In: Keresztesi B (ed) Az akác termesztése és hasznosítása. Mezőgazdasági Kiadó, Budapest, pp 20–24
- Keresztesi B (ed) (1988) The black locust. Akadémiai Kiadó, Budapest
- Kim YK, Lee S, Song JH et al (2020) Comparison of biochemical constituents and contents in floral nectar of *Castanea* spp. *Molecules* 25(18):4225. <https://doi.org/10.3390/molecules25184225>
- Kollipara KP, Hymowitz T (1992) Characterisation of trypsin and chymotrypsin inhibitors in the wild perennial *Glycine* species. *J Agric Food Chem* 40:2356–2363. <https://doi.org/10.1021/jf00024a007>
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685. <https://doi.org/10.1038/227680a0>
- Lian C, Hogetsu T (2002) Development of microsatellite marker sin black locust (*Robinia pseudoacacia*) using a dual-suppression-PCR technique. *Mol Ecol Notes* 2:211–213. <https://doi.org/10.1046/j.1471-8286.2002.00213.x-i2>
- Lukash O, Morskyi V, Kurhaluk N, Tkaczenko H (2024) Black locust (*Robinia pseudoacacia* L.) as a medicinal plant with antibacterial activities. *MOJ Food Process Technol* 12(2):152–154. <https://doi.org/10.15406/mojfpt.2024.12.00312>
- Ma J, Huangfu W, Yang X et al (2022) “King of the forage”—alfalfa supplementation improves growth, reproductive performance, health condition and meat quality of pigs. *Front Vet Sci*. <https://doi.org/10.3389/fvets.2022.1025942>
- Makkar HPS, Becker K (1999) Plant toxins and antinutritional factors in agricultural by-products and their alleviation. *Asian-Australas J Anim Sci* 12(4):588–604
- Minekus M, Alminger M, Alvito P et al (2014) A standardised static in vitro digestion method suitable for food—an international consensus. *Food Funct* 5:1113–1124. <https://doi.org/10.1039/c3fo60702j>
- Mishima K, Hirao T, Urano S, Watanabe A, Takata K (2009) Isolation and characterization of microsatellite markers from *Robinia pseudoacacia* L. *Mol Ecol Resour* 9:850–852. <https://doi.org/10.1111/j.1755-0998.2008.02306.x>
- Móricz Á, Baglyas M, Darcsi A, Balla J, Morlock GE (2024) New antioxidant caffeate esters of fatty alcohols identified in *Robinia pseudoacacia*. *Molecules* 29(23):5673. <https://doi.org/10.3390/molecules29235673>
- Papachristou T (1999) Assessing the value of black locust (*Robinia pseudoacacia* L.) browse for animal feeding
- Peakall R, Smouse PE (2006) GenAlEx 6.4: genetic analysis in Excel. population genetic software for teaching and research. *Mol Ecol Notes* 6:288–295. <https://doi.org/10.1111/j.1471-8286.2005.01155.x>
- Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. population genetic software for teaching and research—an update. *Bioinformatics* 28:2537–2539. <https://doi.org/10.1093/bioinformatics/bts460>
- Rabani M, Melzig MF (2010) Toxicity of *Robinia pseudoacacia*: a review of the biochemical and toxicological aspects. *Phytother Res* 24(12):1735–1744
- Raeeszadeh M, Beheshtipour J, Jamali R, Akbari A (2022) The antioxidant properties of alfalfa (*Medicago sativa* L.) and its biochemical, antioxidant, anti-inflammatory, and pathological effects on nicotine-induced oxidative stress in the rat liver. *Oxid Med Cell Longev* 2022:1–13. <https://doi.org/10.1155/2022/2691577>
- Rakesh DD, Bhat TK, Singh B (2000) Effect of fungal treatment on composition, tannin levels, and digestibility of black locust (*Robinia pseudoacacia*) leaves. *J Gen Appl Microbiol* 46(2):99–103. <https://doi.org/10.2323/jgam.46.99>
- Rédei K, Csiha I, Rásó J, Keserű Z (2017) Selection of promising black locust (*Robinia pseudoacacia* L.) cultivars in Hungary. *J for Sci* 63:339–343. <https://doi.org/10.17221/23/2017-JFS>
- Rodríguez H, de las Rivas B, Gómez-Cordovés C, Muñoz R (2008) Degradation of tannic acid by cell-free extracts of *Lactobacillus plantarum*. *Food Chem* 107(2):664–670. <https://doi.org/10.1016/j.foodchem.2007.08.063>
- Samsonova I, Mannapov A, Khisamov R, Plakhova A, Sattarov V (2020) Analysis of honey productivity of robinia (*Robinia pseudoacacia* L.) plantations in forest vegetation

- conditions of the steppe don region. IOP Conf Ser Earth Environ Sci 574(1):012071. <https://doi.org/10.1088/1755-1315/574/1/012071>
- Semwal RB, Semwal DK, Combrinck S, Trill J, Gibbons S, Viljoen A (2019) Acacetin—a simple flavone exhibiting diverse pharmacological activities. *Phytochem Lett* 32:56–65. <https://doi.org/10.1016/j.phytol.2019.04.021>
- Singleton VL, Orthofer R, Lamuela-Raventos RM (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol* 299:152–178. [https://doi.org/10.1016/S0076-6879\(99\)99017-1](https://doi.org/10.1016/S0076-6879(99)99017-1)
- Snyder LJU, Luginbuhl JM, Mueller JP, Conrad AP, Turner KE (2006) Intake, digestibility and nitrogen utilization of *Robinia pseudoacacia* foliage fed to growing goat wethers. *Small Rumin Res* 71(1–3):179–193. <https://doi.org/10.1016/j.smallrumres.2006.06.006>
- Tian F, McLaughlin JL (2000) Bioactive flavonoids from the black locust tree, *Robinia pseudoacacia*. *Pharm Biol* 38(3):229–234. [https://doi.org/10.1076/1388-0209\(200007\)3831-SFT229](https://doi.org/10.1076/1388-0209(200007)3831-SFT229)
- Tóth-Márkus M, Sass-Kiss Á (1993) Effect of cooking on the fatty acid composition of silver carp (*Hypophthalmichthys molitrix*, V.). *Acta Aliment* 22:25–35
- Varga A (2017) Innovation from the past: Silvopastoral systems in Hungary in the light of Hungarian ethnographic literature. *Acta Ethnogr Hung* 62(1):135
- Vlaicu PA, Untea AE, Turcu RP, Saracila M, Varzaru I, Oancea AG (2023) Chemical composition of dietary alfalfa and its effectiveness on broiler chicken thigh meat quality. *Czech J Food Sci* 41(4):279–286. <https://doi.org/10.17221/79/2023-CJFS>
- Weising K, Nybom H, Wolff K, Kahl G (2005) DNA fingerprinting in plants: principles, methods and applications, 2nd edn. CRC Press, Boca Raton
- Wina E, Tangendjaja B, Dumaria D (2008) Effect of Calliandra calothyrsus on in vitro digestibility of soybean meal and tofu wastes. *Livest Res Rural Dev* 20(6):98
- Zang G, Mi W, Li Y, Jiang J, Lu C, Niu C (2010) Effects of different supplements on tetraploid black locust (*Robinia pseudoacacia* L.) silage. *For Stud China* 12(4):176–183
- Zarev Y (2023) Isolation and characterization of 3-O-caffeoyloleanolic acid from *Robinia pseudoacacia* stem bark. *Pharmacia* 70(4):1209–1212. <https://doi.org/10.3897/pharmacia.70.e110402>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.