Doctoral dissertation

Effect of dietary Paulownia leaves on ruminal methanogenesis and biohydrogenation in dairy cows

Liści Paulowni w regulacji metanogenezy i biouwodorowania u krów mlecznych

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MEiN = 200 pct
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6. Smulski, S., Gehrke, M., Libera, K., Cieslak, A., Huang, H., Patra, A. K., & Szumacher-Strabel, M. (2020). Effects of various mastitis treatments on the reproductive performance of cows. BMC veterinary research, 16, 1-10.

ISI impact factor (2021) = 2.670; ISI impact factor 5 years = 2.699

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7. Gao, M., Cieślak, A., **Huang, H**., Gogulski, M., Petrič, D., Ruska, D., ... & Szumacher-Strabel, M. (2023). Effects of raw and fermented rapeseed cake on ruminal fermentation, methane emission, and milk production in lactating dairy cows. *Animal Feed Science and Technology*, *300*, 115644.

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Abstract

Paulownia is a genus of fast-growing trees that generate a huge mass of leaves, which can be utilized as a feed resource for ruminants. In the present study, the chemical and phytochemical composition, *in vitro* and *in vivo* ruminal fermentation, methane production, and feasibility of fresh Paulownia hybrid leaves (PL) and its silage (PLS) were investigated based on commercial farm conditions. The crude protein content of PL and PLS ranged from 132 to 199 g/kg dry matter, which was comparable to alfalfa silage (AS). Ensiling of PL increased the amount of both phenolic acids and flavonoids. The amino acid content increased, and total saturated fatty acid concentration tended to increase in PLS compared with PL. In the *in vitro* study with the ruminal fluid (batch culture), PL and PLS decreased pH ($P < 0.01$) and methane emission compared with AS. Total archaea counts were lowest in PLS, intermediate in PL, and highest in AS. *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens*, and *Prevotella* spp. were higher ($P \leq 0.03$) in the PL and PLS than in the AS group. The total gas production and total volatile fatty acid concentrations were higher ($P < 0.01$) in both PL and PLS than in AS. The concentrations of acetate in PL and propionate in PL and PLS were greater than those in AS. The potential degradability of DM was higher ($P < 0.01$) for PL and PLS than for AS. PLS had significantly the greatest potential degradability and effective degradability compared to the other groups ($P < 0.001$). Several variables of the ruminal fluid were changed in response to the inclusion of PLS. The addition of PLS also affected several populations of the analyzed microorganisms. The abundance of protozoa and bacteria was increased, whereas the abundance of archaea was decreased by PLS. Methane production decreased by 11% and 14% in PLS-fed cows respectively. In the PLS-fed cows, a reduction in the milk protein and lactose yield was observed, but no effect on DMI and energy-corrected milk yield. Also, the PLS diet affected the ruminal biohydrogenation process with an increased proportion of C18:3 cis-9 cis-12 cis-15, conjugated linoleic acid, C18:1 trans-11 FA, polyunsaturated fatty acids (PUFA), and reduced n6/n3 ratio and saturated fatty acids (SFA) proportion in milk. The relative transcript abundances of 5 of 6 analyzed genes regulating FA metabolism increased.

Key words: Paulownia leaves, nutritive value, phenolic acids, flavonoids, milk quality, rumen

Streszczenie

Paulownia to rodzaj szybko rosnących drzew, które generują ogromną masę liści, które można wykorzystać jako źródło paszy dla przeżuwaczy. W pracy zbadano skład chemiczny i fitochemiczny, fermentację w żwaczu *in vitro* i *in vivo* oraz produkcję metanu, a także przeprowadzono analizę chemiczną liści paulowni w formie świeżej (PL) oraz kiszonki (PLS) w odniesieniu do warunków plantacji. Zawartość białka ogólnego w PL i PLS wahała się od 132 do 199 g/kg suchej masy, co było porównywalne z wartością pokarmową kiszonki z lucerny (AS). Proces kiszenia PL zwiększył ilość zarówno kwasów fenolowych, jak i flawonoidów. Zawartość aminokwasów wzrosła, a całkowita koncentracja nasyconych kwasów tłuszczowych wykazywało tendencję do wzrostu w PLS w porównaniu z PL. W badaniu *in vitro* (batch culture) PL i PLS zmniejszyły wartość pH ($P<0,01$) w płynie żwacza po inkubacji oraz przyczyniły się do redukcji emisji metanu w porównaniu z AS. Całkowita liczba metanogenów była najniższa w PLS, pośrednia w PL, a najwyższa w AS. *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens* i *Prevotella* spp. były statystycznie istotnie wyższe ($P\leq0,03$) w grupie PL i PLS niż w grupie AS. Całkowita produkcja gazów i całkowita koncentracja lotnych kwasów tłuszczowych były statystycznie istotnie wyższe ($P<0,01$) zarówno w grupie PL, jak i PLS niż w grupie AS. Stężenia kwasu octowego w PL oraz kwasu propionowego w PL i PLS były wyższe niż w AS. Rozkład suchej masy w żwaczu był wyższy ($P<0,01$) dla PL i PLS niż dla AS. PLS cechowało się wyższym efektywnym rozkładem białka w żwaczu ($P<0,001$). Zastosowanie PLS w dawkach pokarmowych dla krów mlecznych wpłynęło na zmiany w wskaźnikach biochemicznych płynu żwacza. Liczebność pierwotniaków oraz bakterii zwiększyła się po zastosowaniu dodatku PLS, podczas gdy PLS zmniejszył liczebność metanogenów. Produkcja metanu zmniejszyła się odpowiednio o 11% i 14% u krów żywionych dawką pokarmową z dodatkiem PLS. Odnotowano zmniejszenie ilości dziennej produkcji białka i laktozy w mleku krów żywionych dawką pokarmową z PLS, ale brak wpływu na wydajność. Ponadto, dawka pokarmowa z PLS wpłynęła na proces biouwodorowania w żwaczu, zwiększając proporcje C18:3 *cis*-9, *cis*-12, *cis*-15, sprzężonego izomeru kwasu linolowego, C18:1 *trans*-11 FA, wielonienasyconych kwasów tłuszczowych (PUFA) i obniżoną zawartość n6/n3 oraz zawartość nasyconych kwasów tłuszczowych (SFA) w mleku. Zanotowano wzrost względnego poziomu transkryptów w 5 z 6 analizowanych genów regulujących metabolizm FA.

Słowa kluczowe: liście paulowni, wartość odżywcza, kwasy fenolowe, flawonoidy, jakość mleka, żwacz

Abbreviations

AS: alfalfa silage; PL: fresh Paulownia leaves; PLS: ensiled Paulownia leaf; DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; NFC: nonfiber carbohydrates; NDF (aNDFom):NDF assayed with a heat stable amylase and expressed exclusive of residual ash; ADF (ADFom): ADF expressed exclusive of residual ash; GE: gross energy; SPE: solid phase extraction; UHRMS: ultra-high-resolution mass spectrometry; FAME: fatty acid methyl esters; CLA: conjugated linoleic acids; GC: gas chromatograph; IVDMD: *in vitro* dry matter digestibility; FISH: fluorescence in situ hybridization; PD: potential degradability; ED: effective rumen degradability; AA: amino acids; EAA: essential amino acids; NAA: nonessential amino acids; FA: fatty acids; SFA: saturated fatty acids; UFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; VFA: volatile fatty acids; PPGs: phenylpropanoid glycosides; A/P ratio: acetate/Propionate ratio; BAC: biologically active compounds; BH: biohydrogenation; CH_4: methane emission; CO_2: carbon dioxide emission; DΔ: desaturation Δ at –n; DI: desaturation index; DMD: dry matter digestibility; DMI: dry matter intake; DNA: deoxyribonucleic acids; EI: elongase index; ELOVL5: fatty acid elongase 5; FADS1: fatty acid desaturase 1; FASN: fatty acid synthase; LA:α-linoleic acids; LCFA: long chain fatty acids; LT: longissimus thoracis; LNA: α-linolenic acids; LPL: lipoprotein lipase; MCFA: medium chain fatty acids; mRNA: messenger-RNA; NDFD: neutral detergent fibre digestibility; NH_3: ammonia; PBS: phosphate-buffered saline -Paulownia leaves silage; PLS60: Paulownia leaves silage- diets containing 60 g/kg DM of PLS; qPCR: quantitative PCR; RA: rumenic acid; RT: transcription reaction; Rusitec: rumen simulation technique; SCD: stearoyl-CoA desaturase; SPE: solid phase extraction; TI: thrombogenicity; VA: vaccenic acid

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1 Introduction

1.1 Animal husbandry production and environmental impact

Animal husbandry is an important component of world food production chains as well as play an important role in the development of the national economy of many developing and developed countries. Animal husbandry has a diverse impact on climate protection, economic development, income resources, and sustainable food security (Bizzuti et al., 2023). From a production perspective, animal husbandry production requires a large amount of feed, such as corn, soybeans, wheat bran, etc. These feeds are not only used for feeding livestock but also for growing crops in agriculture (Qian and Cuixia 2012). From this point of view, the development of animal husbandry has promoted the development of agriculture and feed production, improving the efficiency and scale of food production (Eilers et al., 2010). In the process of animal husbandry development, a large area of grassland and pasture is required. These grasslands and pastures can improve the production capacity and quality of land, thereby increasing the yield and quality of world food production from the perspective of land productivity (Tulu et al., 2023). Dairy products (milk and meat) produced by animal husbandry are important sources of high-quality proteins generally considered essential for human well-being (Leroy et al., 2022). Furthermore, animal-source proteins fulfill 45-60% of human protein requirements (Vieux et al., 2022). It is predicted that global annual meat production will increase to over 400 million tons by 2050. In this sense, animal husbandry is the full utilization of resources and the best way to supplement food production.

Animal husbandry requires a large amount of land for raising animals and producing feed. This has led to extensive deforestation, grassland degradation, and loss of land productivity (Scholtz et al., 2013). This not only damages the ecosystem but also leads to a shortage of land resources and environmental pollution (Oyekale 2014). Animal husbandry is one of the main sources of global greenhouse gas emissions, especially methane and carbon dioxide. It is estimated that animal husbandry contributes approximately 14.5% of global anthropogenic greenhouse gas emissions (Gerber et al., 2013;Nin-Pratt et al., 2022). This has an undeniable impact on climate change. In addition, animal husbandry requires a large number of water resources for drinking, cleaning, and production of feed. However, many regions around the world are facing water scarcity issues, so the demand for water resources in animal husbandry may lead to excessive use

and pollution of water resources, which also contradicts the requirements of sustainable development (Gjerris et al., 2011). Another factor that is not conducive to sustainable development is health risks. There are also risks related to animal health in animal husbandry, such as outbreaks of diseases and drug residues. These issues not only affect the production capacity of animals but also pose a threat to human health.

Animal husbandry plays an irreplaceable role in world food production, but it also poses important challenges to sustainable development and climate change in its development process. To address these challenges, a series of measures need to be taken, including promoting sustainable animal husbandry production methods, reducing greenhouse gas emissions, improving water resource utilization efficiency, and strengthening animal health monitoring and management. Animal husbandry is a pillar of food security and rural livelihoods. Therefore, the international community must work together to ensure that the industry can unleash its potential and contribute to sustainable development. In order to ensure the sustainable growth of animal husbandry production, the world should further promote the efficiency of natural resource utilization in animal husbandry to avoid excessive pressure on ecosystems, biodiversity, land and forest resources, water quality, and global warming (Ponnusamy and Pachaiyappan 2018).

1.2 Ruminal fermentation and methanogenesis

Ruminal metabolism in ruminants refers to the fermentation and biohydrogenation of feed by microorganisms in the rumen. There is a type of microorganism called methanogens in the rumen that can utilize hydrogen and carbon dioxide to produce methane. These microorganism mainly exist in the posterior chamber and folds of the rumen, and only grow under hypoxic conditions (Pal et al., 2015). Therefore, when the oxygen level in the rumen decreases, these bacteria start to produce methane. Ruminants are capable of converting fibrous plant materials that cannot be directly utilized by humans into high-quality livestock products such as meat and milk (Greening et al., 2019). Through ruminal microorganisms, cellulose and other non-digestible carbohydrates can be converted into easily digestible substances to improve nutrient utilization (Ahmed et al., 2021). The microorganisms in the rumen include bacteria, fungi, and protozoa. Nutrients in the feed are degraded by microorganisms in the rumen to produce volatile fatty acids, peptides, amino acids, and ammonia. Rumen microorganisms can also use

nitrogen sources to synthesize microbial protein, B vitamins, vitamin K and other nutrients for ruminant utilization (Kim et al., 2020).

However, ruminal fermentation also involves the generation and emission of greenhouse gases such as methane. According to statistics, ruminants emit about 100 million tons of methane into the environment every year, accounting for about one-fifth of the global total annual methane emissions (Kumar et al., 2015). In the rumen of ruminants, fiber-degrading bacteria, methanogens, and other anaerobic microorganisms degrade cellulose in the forage and produce various organic acids and alcohols through fermentation. These simple organic compounds can be used as carbon sources by methane-producing bacteria. Therefore, fermentation processes similar to those in biogas digesters are also carried out in the rumen of cows and other ruminants. During this process, beneficial products of microbial metabolism can be directly absorbed and utilized by ruminants, while gases such as CH_4 and CO_2 produced during fermentation are expelled from the mouth during the regurgitation process (Shibata and Terada 2010). Experiments have shown that the rumen of an adult cow can hold about 100 liters of fermentation products, and methane can reach 200 liters per day in the gas-discharged (McAllister and Newbold 2008).

The production of methane is the result of the combined action of various microorganisms in the rumen. Among them, the most direct methane producer is methane-producing archaea. Other microorganisms include protozoa, bacteria, and fungi. Scholars classify protozoa in the rumen into two categories based on their structure and activity, namely *Holotricha* protozoa and *Entodiniomorpha* protozoa. *Holotricha* protozoa, whose surface membrane is completely covered by cilia, mainly digest soluble substrates; *Entodiniomorpha* protozoa have a hard surface membrane and cilia located only near the “mouth”, capable of digesting particulate matter (Belanche et al., 2014). Rumen protozoa produce a large amount of hydrogen for hydrogenotrophic methanogenic archaea through their hydrogenase activity, so they are also important participants in methane production. *Metanobrevibater* and *Metanomicrobium* are considered to be the two main methanogenic archaea genera that have a symbiotic relationship with protozoa (Janssen and Kirs 2008). Similar to protozoa, fungi are also involved in the methane production process (Patra and Saxena 2010). As fungal fermentation produces hydrogen as a substrate for methane production and other metabolic end-products such as carbon dioxide, formic acid, and acetic acid (Akash et al., 2023).

In rumen metabolism, an efficient biotransformation process, ruminants use microorganisms to decompose and ferment complex carbohydrates to produce short-chain fatty acids and gases, including methane. This process provides energy and nutrition for ruminants, while also having an impact on the environment.

1.3 Effect of plant bioactive substances on ruminal fermentation

Plants contain a large amount of bioactive substances, such as tannic acid, flavonoids, tannins, volatile oils, alkaloids, saponins, etc. (Aka et al., 2011). These compounds can affect the rumen metabolism of ruminants, by their inhibitory or stimulating effects on the growth and metabolism of rumen microorganisms (Gry et al., 2001).

Tannin is a polyphenolic compound widely present in plants, with strong oxidation-reduction ability, including tannic acid, catechins, etc. It is commonly found in plants such as tea, grapes, and oak. In addition to its antioxidant function, it also has biological activities such as antibacterial activity. Some tannin compounds can bind to proteins in the rumen, forming complex compounds that are difficult to digest, thereby affecting the speed of food passage in the rumen and the growth and metabolism of rumen microorganisms (Cieslak et al., 2013). Tannic acid is a common secondary metabolite in plants, commonly found in bark, nuts, fruits, and, vegetables. It has biological activities such as astringency, and antibacterial, and antioxidant properties (Bahromovich).

Flavonoids are commonly found in plants such as beans, tea, fruits, and vegetables (Ribeiro et al., 2023). Dietary flavonoids demonstrated an improvement in growth, health, feeding efficiency, and meat quality as well as antimicrobial, and antioxidant bioactivities in ruminants (North et al., 2019). Some flavonoids can inhibit the growth of methanogens in the rumen, thereby reducing methane production.

Essential oils are also belonging to secondary metabolites of plants, commonly found in aromatic plants, including menthol, camphor, eugenol, etc. It has analgesic, antibacterial, anti-inflammatory and other biological activities. Some compounds in essential oil can promote the growth and metabolism of rumen microorganisms, thereby increasing the digestive capacity of ruminants (Becker et al., ;Elghandour et al., 2023).

Alkaloids are natural compounds containing nitrogen, including morphine, caffeine, solanine, etc., (Bribi 2018). They are commonly found in various plants and have biological activities such as analgesia, nerve stimulation, and antibacterial activity.

Some alkaloid compounds can inhibit the growth and metabolism of microorganisms in the rumen, thereby reducing the digestive capacity of ruminants.

Saponins are widely distributed in plants (Li et al., 2023). They have biological activities such as reducing blood lipids, antibacterial properties, and promoting digestion. Some compounds in saponins can inhibit the growth and metabolism of microorganisms in the rumen, thereby reducing the digestive capacity of ruminants.

Researchers have summarized the impact of bioactive compounds in the following aspects. Promoting rumen fermentation: (1) plant polysaccharides can modulate the ruminal fermentation by increasing the total volatile fatty production, and propionate proportions (Li et al., 2018); (2) inhibition of rumen microorganisms: some bioactive substances in plants, such as tannic acid, flavonoids, saponins, etc., have antibacterial and antioxidant effects. As a result, can inhibit the growth and metabolism of rumen microorganisms, and reduce methane and other gas production (Bhatta et al., 2013;Nowak et al., 2022); (3) improving the rumen environment: some bioactive substances in plants, such as essential oils and polysaccharide compounds, can improve the rumen environment (Medjekal et al., 2017), enhance the growth and metabolic ability of rumen microorganisms, and thereby increase the production of rumen fermentation products; (4) adjusting the pH value of the rumen: Some bioactive substances in plants, such as tannins and tannic acid, can regulate the pH value of the rumen and maintain it within an appropriate range, thereby promoting the growth and metabolism of rumen microorganisms (Delgado et al., 2010).

Some bioactive substances in plants have functions such as regulating the proportion of volatile fatty acids in the rumen, by modulating the structure of rumen microbiota and therefore reducing methane emissions (Nowak et al., 2022). The impact of plant bioactive substances on rumen is complex, and there may be interactions and influences between different substances. Therefore, the response of rumen to bioactive substances varies among different species.

1.4 Bioactive substances in Paulownia leaves

Paulownia tree is a deciduous tree with multiple varieties. This type of plant is commonly found in Asia, Europe, and North America. They can grow very tall, with a tree height of 20-30 meters and a trunk diameter of over 1 meter (Guo-qiang et al., 2001). The bark of the Paulownia tree is smooth, grey, or greyish brown, with a sturdy trunk and

a relatively broad crown. The leaves of Paulownia trees are large and broad, heart-shaped or ovoid, 20 to 40 centimeters long and 15 to 30 centimeters wide, green or dark green, with smooth and obvious veins (Kuznicki et al., 2018). The flowers of Paulownia trees are usually bell-shaped and brightly colored, often used for landscaping or ornamental purposes (Morote et al., 2023). There are various species of Paulownia trees, mainly purple Paulownia, white Paulownia, flower leaf Paulownia, short-order Paulownia, etc. These tree species have a wide range of growth environments and can adapt to different climates and soil conditions, so they are widely planted and utilized in different regions. Paulownia trees are not only used for landscaping and ornamental purposes, but also for wood, papermaking, medicine, food, and other purpose (Bodnár et al., 2014).

After the Paulownia flower shedding, the leaves gradually become expand (Stanković et al., 2009). Paulownia leaves contain nutrients such as crude fat and protein, as well as important elements such as iron, manganese, and zinc (Bodnár et al., 2014). Therefore, for feeding ruminants, Paulownia leaves are not only abundant in basic nutrients but also have sufficient amounts of bioactive compounds. In 1931, glycoside compounds were isolated from the bark and leaves of Paulownia leaves. With the development of science and technology, various chromatographic separation methods and modern spectroscopic techniques have been applied to the study of natural products, and new compounds have been continuously discovered from Paulownia plants (Tahama 1980;Stochmal et al., 2022).

Its leaves contain various bioactive compounds. Paulownia leaves contain a large amount of nitrogen glycosides such as adenosine, guanosine, and cytidine, which have the effects of regulating immune function, lowering blood pressure, and improving blood circulation. Polysaccharides present in Paulownia leaves, such as arabinose, xylose, galactose, etc., can resist oxidation, lower blood glucose and have anti-tumor properties (Woźniak et al., 2022). It contains a variety of flavonoids, such as rhamnoside and rutin, which have antioxidant, anti-inflammatory, and blood pressure-lowering effects. Cellulose-rich Paulownia leaves can promote intestinal peristalsis and increase satiety in ruminants. In addition, the essential oil present in Paulownia leaves is mainly composed of eucalyptol and eucalyptol, which have bactericidal, anti-inflammatory, and insect-repellent effects (Bodnár et al., 2014). It should be noted that the bioactive substances in Paulownia leaves have certain toxic effects and should not be consumed excessively. As excessive intake can exert a negative impact on the health of ruminants (Bodnár et al., 2014).

1.5 Paulownia leaves as a dietary component in ruminant nutrition

The dietary components required by ruminants include crude protein, carbohydrates, fats, vitamins minerals. Ruminants require sufficient energy to maintain normal physiological metabolism, growth, and development. The main energy sources are cellulose (Nawaz and Ali 2016). Amino acids are building blocks of proteins vital for body growth, development, maintenance, health, lactation, and reproduction in ruminants (Alvarado 2019). Meanwhile, ruminants require an appropriate amount of minerals and trace elements including calcium, phosphorus, potassium, magnesium, copper, zinc, selenium, etc., to maintain normal physiological functions. Vitamins are also essential nutrients for the growth and health of ruminants, including vitamins A, D, E, K, and B vitamins. In addition to the above basic nutrients, ruminants also need some special ingredients to promote rumen fermentation, such as cellulose, and hemicellulose. In addition, a certain proportion of crude fiber and non-starch polysaccharides are also required in the feed of ruminants to promote gastrointestinal peristalsis and digestive functions (Bryszak et al., 2020).

Paulownia leaves can provide ruminants with the necessary nutrients such as protein, fat, and fiber, which are beneficial for their normal growth and development. On the other hand, the bioactive components in Paulownia leaves can enhance the immunity and disease resistance of ruminants, thereby reducing their risk of disease (Lata and Mondal 2021). For example, the polysaccharides in Paulownia leaves can enhance the immune and antioxidant abilities of ruminants, while also having certain anti-inflammatory effects, which are beneficial for promoting the health of ruminants' digestive system.

Paulownia leaves contain about 14%-23% crude protein, 11% crude fiber, 2-4 % crude fat, and 6-9% ash contents (Stewart et al., 2018). They also contain amino acids particularly lysine and methionine, as well as vitamin C (Bodnár et al., 2014;Lata and Mondal 2021). Some studies showed that Paulownia leaves can be used in feeding different species (Alagawany et al., 2022), however, no studies have been performed on high-yielding dairy cows.

2 Hypothesis and Aim

2.1 Hypothesis

We hypothesized that Paulownia leaves with high concentrations of crude protein and bioactive components modulate ruminal fermentation and nutrient degradation, and thus can be considered as a valuable dietary component for dairy cows.

2.2 Aim

The aim was to determine the chemical and phytochemical composition of Paulownia leaves and to investigate the effects of Paulownia leaves on *in vitro* and *in vivo* rumen fermentation characteristics, *in situ* nutrient degradation, ruminal methane production, microbial population, milk production, and their composition.

3 Material and Methods

3.1 Paulownia leaves collection analysis and experimental design

The fresh (PL) and ensiled (PLS) Paulownia leaves used in this study were obtained from *Paulownia tomentosa* × *Paulownia fortunei* hybrid trees. We collected the leaves from two-year-old and three-year-old plantations over two consecutive years (2018 and 2019). During this period, we collaborated with four Polish plantations located near Grzybno (heavy loamy soil, class IV, 1000 trees), Modrze (light permeable soil, class IV, 386 trees), Gierłatowo (sandy soil, class III, 400 trees), and Chorzyna (sandy lands, class IV, 1200 trees). At the end of May when the trees were shaped, we collected eight (four/year × 2 years) representative samples of Paulownia leaves. We collected leaf samples from at least fifty trees of each plantation, analyzed them as fresh and ensiled, and then lyophilized them. Paulownia leaves from each of the 4 plantations were ensiled within 8 h after harvesting with the biological additive Agricol Sil (Microferm, UK) in three plastic microsilos (three replicates per plantation per year). Each consists of plastic drums of 4 dm^3, 15 cm in diameter and 49 cm in height. Thus, 12 microsilos were ensiled each year with a total of 24 microsilos over two years. According to Szumacher-Strabel et al. (2019) ensiling protocol was used. Approximately 2.4 kg of chopped fresh PL was immediately packed into microsilos, sealed with two screw tops (internal and external) and stored at ambient temperature (20-25 °C) for 8 weeks. After the ensiling period, the microsilos were opened, and samples were obtained for chemical analysis. For *in vivo* experiments, 50 plastic drums were prepared by using the same methodology. Samples (representative batch) of the ensiled materials were lyophilized using a freeze-drier (Christ Gamma 2-16 LSC, Martin Christ, Osterode am Harz, Germany) and kept in the dark for phenolic acid and flavonoid analysis, and for *in vitro* and *in situ* experiments. The alfalfa silage (AS) was also used as a control for all of the experiments. The alfalfa was harvested at about the 10% bloom stage from a three-year-old plantation located near Września (clay lands, class III) and 4 samples from 4 different spots of the plantation were collected. Before ensiling, alfalfa was wilted to 300 g/kg DM content and chopped to approximately 1.5 cm particle length. Alfalfa silage (AS) was prepared on a large scale for *in vivo* experiments. Like PL ensiling, the same biological additive in the same dosage was applied and prepared for AS.

Before the *in vitro* (batch culture) and *in situ* (rumen *in sacco*) experiments, all the Paulownia samples were pooled and thoroughly mixed (separately for PL and PLS). One

representative of a homogeneous sample was prepared for each plantation. Finally, 4 samples of PL and 4 samples of PLS were prepared. One sample represented one plantation. Regarding AS silages, one representative and homogeneous sample from each area was prepared. Finally, 4 AS samples were tested. PL and AS were used from plastic drums and large scale for *in vivo* experiments respectively.

3.2 *In vitro* experiments

3.2.1 Batch culture (Experiment 1)

The whole procedure for preparing and running a short-term batch culture fermentation was carried out following the modified procedure described by Cieslak et al., (2016). The rumen inoculum was obtained from three ruminal cannulated Polish Holstein-Friesian dairy cows (body weight 625 ± 25 kg, second month of lactation) before the morning feeding. The rumen inoculum donors were fed 24 kg dry matter (DM) of a total mixed ration (TMR). Alfalfa silage was used as a control to compare with tested feeds (PL and PLS) because of its similarity to PL and AS nutritional value. The experiment was repeated for three consecutive days. Twelve Paulownia (PL, PLS) samples (4 plantations × 3 independent runs) and 4 AS samples (4 areas x 3 independent runs) were analyzed. In rumen fluid, after incubation, the basic rumen fermentation, methane production, and microbial populations were determined.

3.2.2 RUSITEC (Experiment 2)

The *in vitro* second experiment was conducted using the Rusitec system equipped with four fermenters of 1 L volume each, following the procedures described by Szczechowiak et al., (2016). Ruminal fluid and solid digesta for the *in vitro* experiment were collected 3 h before the morning feeding from four rumen-cannulated (Bar Diamond, Parma, Idaho, USA) multiparous Polish Holstein–Friesian dairy cows (630 ± 25 kg body weight) at their 3rd month of lactation. The following diets were tested: a control diet (CON) and three PLS diets. The CON diet contained the following forages: corn silage (388 g/kg DM), alfalfa silage (82 g/kg DM), and meadow grass silage (91 g/kg DM; Table 1). The PLS diets contained Paulownia silage that replaced alfalfa silage at 25%, 50%, and 75%. They corresponded to the PLS content in the diet at the level of 20, 40, and 60 g/kg DM (Table 1). Ruminal fluid donor cows received the CON diet twice a day *ad libitum* (Table 1).

The *in vitro* experiment was designed in a completely randomized design comprising four diets and three replicates. From day 6 to 10 of each run, fermentation fluid samples were collected under anaerobic conditions from each vessel 3 h before feeding time. We analyzed the collected fluid samples for pH, volatile fatty acids (VFA), ammonia concentration, protozoa, bacteria and methanogen counts. For fatty acids analysis, samples were collected directly from the effluent vessels during bag replacements. Before feeding time (once a day), the fermentation gas was collected in a gas-tight collection bag (Tecobag 81; Tesseraux Container, Bürstadt, Germany) for the methane concentration measurement. The DM degradability was determined by analyzing feed residues in pre-feeding nylon bag samples for the last 5 d of each run (d 6 to 10).

Table 1. Ingredients and chemical composition of experimental diets (n = 4) used in Rusitec system and *in vivo* experimets[1] and chemical composition of Paulownia leaves silage (PLS; n = 4) and alfalfa silage (AS; n = 4)

Item	PLS	AS	Treatments [2]			
			CON	PLS (g/kg DM)		
				20	40	60
Ingredient composition, g/kg DM						
Corn silage	-	-	388	385	386	386
Alfalfa silage	-	-	82	68	47	26
Paulownia silage	-	-	0	21	39	60
Meadow grass silage	-	-	91	90	90	90
Beet pulp	-	-	103	103	103	103
Brewer's grain	-	-	95	94	95	95
Concentrate[3]	-	-	119	118	119	119
Rapeseed meal	-	-	108	107	107	107
Mineral and vitamin premix[4]	-	-	14	14	14	14
Forage to concentrate ratio	-	-	76:24	76:24	76:24	76:24
Chemical composition[5], g/kg DM						
DM, g/kg as fed	279	299	425	423	422	419
OM	869	874	909	907	904	900
aNDF	359	350	347	346	349	351
CP	174	218	161	160	158	158
EE	27.5	20.0	27.1	29.8	30.5	29.5
Total phenolic compounds[6]	60.0	-	0.00	1.20	2.40	3.60
VEM[7]	785	667	948	945	943	942
Fatty acid composition, g/100 g total FA						
C12:0	0.19	2.18	0.31	0.24	0.31	0.31
C14:0	0.71	1.90	0.47	0.40	0.50	0.48
C16:0	27.4	26.5	22.3	21.3	21.2	21.5

C16:1 *cis*-9	3.89	1.36	0.74	0.78	1.03	0.99
C18:0	4.16	4.01	2.63	2.55	2.16	2.82
C18:1 *cis*-9	5.26	4.76	20.3	19.7	18.9	17.8
C18:2 *cis*-9, *cis*-12	16.2	20.4	44.4	45.2	44.6	44.7
C18:3 *cis*-9, *cis*-12, *cis*-15	42.2	38.8	8.85	9.83	11.3	11.4

[1]In the in vitro experiments the diets were as a total mixed ration (TMR).
[2]CON: control diet; PLS: Paulownia leaves silage diet; PLS was used at 20, 40 and 60 g/kg DM of diet replacing alfalfa silage.
[3]Declared to contain (as g/kg of DM in concentrate) OM (910), aNDFom (240), CP (17.5), and EE (31).
[4]Declared to contain (g/kg of DM) Na (123), Ca (100), Mg (45), P (42), K (20), S (18), Co (14), Cu (5.0), Zn (2.8), Mn (1.4), Fe (1.05), F (0.42), I (0.028), Se (0.018), biotin (0.008); (IU/kg), vitamin A (200,000), vitamin D3 (40,000), and vitamin E (1,200).
[5]DM: dry matter; OM: organic matter; aNDF: neutral detergent fiber analyzed with α-amylase; CP: crude protein; EE: ether extract. Additionally, in the case of PLS and AS, the following fermentation parameters were performed: pH – 4.75 vs. 4.50; NH_3-N – 79.4 vs. 32.3 g/kg total nitrogen; lactic acid – 21.2 vs. 34.9 g/kg DM; acetic acid –7.04 vs. 19.1 g/kg DM; propionic acid –1.29 vs. 1.09 g/kg DM; butyric acid – 1.19 vs. 2.43 g/kg DM, respectively.
[6]The content of total phenolic compounds have been calculated based on previous study of Huang et al. (2021).
[7]VEM = feed unit net energy lactation; calculated using the FeedExpert software.

3.3 *In vivo* experiments

3.3.1 *In sacco* (Experiment 3)

Three lactating multiparous Polish Holstein Friesian cows (625 ± 25 kg body weight) fitted with rumen cannulas (10 cm, Bar Diamond, Parma, Idaho, USA) were used. The cows were fed twice daily at 06:00 and 18:00 h, with the TMR consisting of 400 g/kg of alfalfa forage, 300 g/kg of grass forage, and 300 g/kg of concentrate (wheat grain, extracted rapeseed meal, extracted soybean meal, and mineral and vitamin premix). Clean water was available *ad libitum*. PL and PLS were tested and AS was used as a control to compare the tested feeds for their degradability. Samples of 2.75 g DM were placed in each dacron bag (6 × 13 cm; pore size 50 μm) and sealed with insoluble surgical sutures approximately 1 cm below the upper edge. Bags were incubated in the rumen of each cow for 0, 2, 4, 8, 12, 24, 48, and 72 h. The zero-hour bags were not placed in the rumen but were treated with distilled water at 39 °C for 15 min. For each incubated feed (AS, PL, or PLS), three cows were used, and the procedure was repeated twice. In total, 240 bags (40 bags × 3 cows × 2 repetitions) were incubated for each feed (24 replicates per PL or PLS, i.e., 3 cows × 4 plantations × 2 runs and 24 replicates per AS, i.e., 3 cows × 4 areas × 2 runs). After the appropriate incubation time, the bags were removed from the rumen and the fermentation of the material in the bags was inhibited by placing the bag in cold water. The potential degradation (PD) and effective rumen degradability (ED) of DM, organic matter (OM), and CP were described.

3.3.2 Cannulated cows (Experiment 4)

Four multiparous cannulated Polish Holstein–Friesian dairy cows (625 ± 20 kg body weight; 4-5th month of lactation) were assigned to two dietary treatments (CON *vs*. PLS60) with two cows in each treatment in a replicated 2 × 2 crossover design. Based on the results from Exp. 1 (mainly pH, total VFA concentration, methane production, and methanogens population), the CON and PLS diets containing the higher level of PLS, i.e., 60 g/kg DM (PLS60) were implemented. Cows were fed two times a day. Each period lasted for 36 d, with a 21-d adaptation and a 15-d sampling period (5 d of rumen fluid collection and 10 d for gases collections). The cows live in tie stalls with rubber mats and individual feeding. They had free access to water and salt blocks during the adaptation and sampling periods. The ruminal fluid was collected from each cannulated cow as described above. Rumen samples (about 400 g/animal) were filtered through a two-layer cheesecloth and analyzed for pH value, ammonia and VFA concentrations, and FA profile. For protozoa counting, about 100 g/animal of the rumen content was mixed with an equal amount of 8% formaldehyde solution (w/w), strained through a two-layer cheesecloth into 10 mL polypropylene tube with a screw cup. Quantification of total bacteria and methanogens was carried out only on rumen fluid sampled 3 h after morning feeding. For microbial analysis, rumen content (300 g) was strained through a two-layer cheesecloth into 100 mL polypropylene box, mixed, transferred into two cryotubes of 4.5 mL, and frozen in liquid nitrogen. Samples were stored at − 80 °C until further analyses.

Feed intake, feed residue, and amount of feces were recorded daily from individual cows kept in respiratory chambers (SPA System, Wroclaw, Poland). This was done during the sampling period (d 27 to 36) to determine the total-tract degradability coefficients.

3.3.3 Commercial dairy cows (Experiment 5)

We assigned 16 multiparous lactating Polish Holstein–Friesian dairy cows to two dietary treatments (CON vs. PLS60). They were having 600 ± 30.4 kg body weight, 2.4 ± 0.45 parity, 160 ± 32 d in milk, and 33 ± 2.1 kg/d milk production; (mean ± SD). Each treatment has eight cows in a replicated 2 × 2 crossover design. Each period consisted of a 21-d adaptation period followed by a 5-d sampling period with a total of 26 d. Cows were randomly assigned to one of the two dietary groups (n = 8): control (CON) and experimental diet (PLS 60). They were kept separately in a designated area of a barn. The two groups (8 CON and 8 PLS60) had separated controlled access to a

computer-controlled feeder station (De Laval, type FP 204, Tumba, Sweden) where concentrate was served. The rest of the diet was offered twice a day (at 06:00 and 18:00 h) as a partial mixed ration (PMR) in individual feeding boxes located on the feeding table. The control PMR contained (g/kg of DM): corn silage (441); alfalfa silage (93), meadow grass silage (103); beet pulp (118), brewer's grain (108); rapeseed meal (122); mineral and vitamin premix (16) whereas experimental PMR contained (g/kg of DM): corn silage (438); alfalfa silage (29), Paulownia leaves silage (68), meadow grass silage (102); beet pulp (117), brewer's grain (108); rapeseed meal (122); mineral and vitamin premix (16). Cows had access to clean water *ad libitum*. The dry matter intake was measured daily for the last 5 d (d 22 to 26) of the experiment. Feces were individually collected from each cow directly after defecation and the floor was kept clean. Cows were milked twice a day at 5:30 and 17:30 h. Milk samples were collected from all cows at each milking during the sampling period (d 22 to 26) for milk basic constituents, FA analysis, and for gene expression analysis.

3.4 Sample analysis

3.4.1 Chemical analysis

Samples of AS, PL and PLS were analyzed following AOAC methods (Latimer 2007). The organic matter (OM) content was calculated from the differences between dry matter (DM) and ash contents. The concentrations of neutral detergent fiber (NDF) and acid detergent fiber (ADF) in feed samples were determined as described by Van Soest et al., (1991). Gross energy (GE) content was determined using an adiabatic bomb calorimeter (KL 12 Mn, Precyzja-Bit PPHU, Bydgoszcz, Poland). Starch concentration was determined by the polarimetric method, following Polish Standard PN-R-64785:1994, and using a polarimeter POL-S2 (Polygen, Wrocław, Poland). The concentration of non-fiber carbohydrates (NFC) was calculated. The silage pH was determined using a pH meter (Elmetron, Type CP-104, Zabrze, Poland). Ammonia was estimated according to the Nessler method by (Szumacher-Strabel et al., 2002). Lactic, acetic, propionic, and butyric acids were determined using a High-Performance Liquid Chromatography (Waters 2690, Santa Clara, CA, USA) equipped with Waters 2487 Dual λ detector and Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, Warsaw, Poland). We immediately measured the pH of ruminal fluid after sample collection using a pH meter (Elmetron, Type CP-104, Zabrze, Poland). The ammonia concentration was

determined with the colorimetric Nessler method and the VFA was analyzed by gas chromatography (GC Varian CP 3380, Sugarland, TX, USA) as described earlier (Szumacher-Strabel et al., 2002;Szczechowiak et al., 2016). In the first two experiments, the content of DM degradability (g/kg) was determined.

3.4.1.1 Analysis of phenolic acids, flavonoids, and saponins

Plant samples (PL and PLS) preparation and extraction were carried out as described previously by Petrič et al., (2020). Chromatographic separation, operating parameters, collision energy, and spectra processing of determination of saponins were performed in line with a previously published protocol (Szumacher-Strabel et al., 2019) using a BEH C18 column (2.1 × 100 mm, 1.7 μm, Waters). We triplicated all the analyses performed in this study.

3.4.1.2 Analysis of amino acid composition and fatty acids profile

The samples of PL and PLS were hydrolyzed with 6 M HCl at 110 °C for 23 h. An Aquity UPLC system (Waters, Milford, MA, USA) equipped with a photodetector was used to analyze amino acid composition as described previously by Tomczak et al., (2018). The fatty acid (FA) profile was analyzed and identified by Szczechowiak et al., (2016). The desaturase index (DI) was calculated as described by Bryszak et al., (2020).

3.4.1.3 Ruminal fermentation parameters and mRNA expression in milk

In the *in vitro* experiment the total gas production and methane concentrations were determined after 24 h of incubation (Cieslak et al., 2016). The measurement of methane production *in vivo* experiment was determined using a gas chromatograph (SRI PeakSimple 310; Alltech, PA, USA) according to Szumacher-Strabel et al., (2011). In the *in vivo* experiments, we measured the CH_4 and CO_2 concentration using two separate NDIR (nondispersive infrared spectroscopy) systems (one system per gas) operating in the near-infrared spectrum (detector 1210 Gfx Servomex 4100, Servomex, Crowborough, UK). In Exp. 4, we used two respiration chambers for monitoring methane production. Specifically, two open-circuit respiration chambers (W × L × H: 300 cm × 400 cm × 220 cm; SPA System, Ltd., Wroclaw, Poland) were used to determine CH_4 and CO_2 over 10 d (d 27 to 36). In the commercial farm experiment, the methane concentration was

measured during the feeding of concentrate in the feeder station for the last 4 d of the experiment. The volatile fatty acid (VFA) concentrations were determined by gas chromatography (GC Varian CP 3380, Sugarland, TX, USA) following a method described by Bryszak et al., (2019). We measured the pH of the rumen samples immediately after sample collection using a pH meter (Type CP-104, Elmetron, Zabrze, Poland). Ammonia concentration was determined using the colorimetric Nessler method (Cieslak et al., 2016). Protozoa in the fermented fluid were counted under a light microscope (Primo Star 5, Zeiss, Jena, Germany) using an appropriate volume (10 µl for *Entodiniomorpha* and 100 µl for *Holotricha*). Methanogen and total bacterial abundances were quantified by fluorescence in situ hybridization (FISH) following the procedure described previously by Szczechowiak et al., (2016). For bacteria quantification, total DNA was extracted from the fermented fluid using QIAamp DNA Stool mini kit (Qiagen GmbH, Hilden, Germany) according to Szczechowiak et al., (2016). Sequences of primers specific to seven selected species and one genus of rumen bacteria are presented in the publication of Huang et al., (2022). Bacteria quantification was performed with a QuantStudio 12 Flex PCR system (Life Technologies, Grand Island, NY, USA) as described previously by Szczechowiak et al., (2016).

The daily milk yields were recorded using a milk meter (WB Ezi-Test Meter 33 kg; True-Test, Manukau, New Zealand). The milk composition was measured by infrared analysis (MilkoScan 255 A/S N, FossElectric, Hillerød, Denmark). For gene expression in milk somatic cells, total RNA was isolated from 10 mL milk samples frozen in liquid nitrogen using a previously published procedure followed by Szczechowiak et al., (2016) The mRNA expressions of six genes encoding enzymes regulating FA metabolism [acetyl-CoA carboxylase 1 (ACACA), fatty acid synthase (FASN), lipoprotein lipase (LPL), stearoyl-CoA desaturase (SCD), fatty acid desaturase 1 (FADS1) and fatty acid elongase 5 (ELOVL5)] were measured in milk somatic cells using previously published primer pairs (Vahmani et al., 2014;Urrutia et al., 2015). Each gene was analyzed in technical duplicates using a LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland) and a LightCycler 480 Sybr Green I Master reagent (Roche Diagnostics, Basel, Switzerland).

3.5 Calculations and statistical analysis

We determined the in-situ degradation kinetics of DM, OM, and CP with the following nonlinear model according to Ørskov and McDonald (1979):

$Dt = a + b \times (1 - e^{-ct})$

Where Dt is the degradation of nutrient at time t, a is the soluble fraction (fraction washed out at t = 0, obtained from bags incubated for 0 h and corrected for particle loss); b is an insoluble degradable fraction; c is the fractional degradation rate (per hour); and t is the time (h).

The effective degradability (ED) of DM, OM, and CP was determined using the following equation:

$ED = a + (b \times c/ (c + k))$

In this formula, k is the ruminal fractional flow rate of 0.06/h.

The chemical composition of PL (n = 8) and PLS (n = 24) were tested with an independent t-test, where the means of both groups were compared using the PROC TTEST procedure. In the batch culture experiment, the data were analyzed with one-way ANOVA taking treatment (i.e., feed type AS, PL, and PLS) as the main factor (n = 12 per feed type). Differences between treatments were tested using Tukey's post-hoc test. All the data from the *in sacco* degradation rate, such as DM, OM, and CP, were subjected to statistical analysis using the one-way ANOVA taking treatment (i.e., feed type AS, PL, and PLS) as a main factor (n = 24 per feed type). The least-square means and Tukey's post-hoc tests were used to compare the differences between means. Data were taken as statistically different when P=<0.05. All values are shown as group means with pooled standard errors of means. The data of the Rusitec experiment were analyzed using a mixed model procedure (PROC MIXED) of SAS (university edition, version 9.4; SAS Institute, Cary, NC, USA). The dietary treatment was considered as the fixed effect, the experimental run as the random effect, and the day (6 to 10 d) as the repeated factor. The linear, quadratic and cubic contrasts were used to determine the effect of the PLS dose. In the cannulated cow experiment, ruminal fermentation and FA data were analyzed using PROC MIXED (ver. 9.4, SAS Institute Inc., Cary, NC) for a crossover design with a model containing group (dietary treatment sequence), period, and treatment as the main effects, sampling time as repeated measures, and cow as a random effect. The model for the bacteria, methanogens and degradability analyses contained cow, group, period, and treatment as

the main effect. In productive dairy cows, the data were subjected to analysis of variance, considering the crossover design, testing the effect of treatment (CON and PLS60), group (dietary treatment sequence), period as fixed effects and cows within a group as a random effect. The analysis of milk production, component yields, and composition was performed on the mean values of the milk variables obtained from two sampling points per day (morning and evening milking). The analysis of FA proportion in the milk, as well as the expression of six genes, was conducted on the mean values of pooled samples from morning and evening milking. The results were tested with an independent t-test where the means of both groups were compared through the PROC TTEST procedure. The results were considered significant when the P-values were less than 0.05. All values are shown as the means with pooled standard errors of means.

4 Results

4.1 Nutrients composition of fresh (PL) and ensiled (PLS) Paulownia leaves

The ensiling process affected some of the analyzed parameters of Paulownia leaves. We noticed a significant decrease ($P < 0.01$) in the starch content and the gross energy value (Table 2) in the Paulownia silage, meanwhile observing a similar nutritive value within PL, PLS, and AS.

Table 2. Chemical composition of alfalfa silage (AS; n = 4), Paulownia leaves (PL; n = 8), and Paulownia leaf silage (PLS; n = 24)

	AS		PL		PLS		
	Mean	SD	Mean	SD	Mean	SD	P-value
Dry matter (g/kg as feed)	292	1.96	275	43.0	232	21.1	0.14
Organic matter (g/kg DM)	870	4.48	886	11.7	875	16.5	0.10
pH	4.56	0.04	—	—	4.81	0.35	—
NH_3-N (g/kg TN)	80.9	5.81	—	—	28.1	9.53	—
Lactic acid (g/kg DM)	35.6	3.17	—	—	20.0	7.88	—
Acetic acid (g/kg DM)	19.0	5.12	—	—	7.29	1.51	—
Propionic acid (g/kg DM)	1.12	0.34	—	—	1.31	0.62	—
Butyric acid (g/kg DM)	3.05	1.58	—	—	1.17	0.33	—
Starch (g/kg DM)	15	3.98	19.6	6.98	12.1	3.99	0.006
Gross energy, MJ/kg DM	15.5	0.54	14.2	0.76	13.2	0.81	0.004

DM: dry matter; TN: total nitrogen; Min: minimum; Max: maximum; SD: standard deviation.

The content of amino acids increased by 22.5 % ($P < 0.003$) for essential AAs and by 23 % ($P < 0.005$) for non-essential AAs (Table 3). Leucine was the most abundant AA in PLS (6.51 g AA/16 g N) whereas phenylalanine dominated in PL (6.82 g AA/16 g N). Considering nonessential AAs, the content of two of them (glycine, asparagine) increased significantly. Glutamine was the most abundant AA in PLS (6.13 g AA/16 g N) whereas serine dominated in PL (5.47 g AA/16 g N).

Table 3. Amino acid (AA) composition (g amino acid/16 g N) of Paulownia leaves (PL; n = 8) and their silage (PLS; n = 24).

	PL				PLS				
	Mean	SD	Min	Max	Mean	SD	Min	Max	P-value
Essential Aas	32.0	2.54	29.0	35.1	39.3	1.56	37.4	40.8	0.003
Nonessential Aas	20.4	1.38	18.7	22.0	25.0	1.63	23.1	26.7	0.005
Total AA	52.4	3.24	49.7	54.1	64.2	3.14	60.6	67.0	0.002

Min: minimum; Max: maximum; SD: standard deviation.

With regard to fatty acid content and composition, fresh Paulownia leaves were enriched in three FAs (C16:0, C18:2 c9, c12, C18:3 c9, c12, c15). Especially in C14:0 (P = 0.017) than PLS (Table 4). The proportion of unsaturated FA (UFA) was approximately twice as high as the saturated FA (SFA) in both groups.

Table 4. Fatty acid (FA) content (mg/g DM) of Paulownia leaves (PL; n = 8) and their silage (PLS; n = 24).

	PL		PLS		
	Mean	SD	Mean	SD	P-value
Total FA	16.2	3.52	17.3	3.67	0.47
C14:0	0.08	0.03	0.11	0.03	0.017
C16:0	3.84	0.49	4.21	0.71	0.13
C18:1 c9	0.73	0.36	0.81	0.4	0.62
C18:2 c9, c12	2.19	0.59	2.5	0.44	0.20
C18:3c9, c12, c15	6.61	2.5	6.58	2.43	0.97
∑ other FA[1]	1.63	0.18	1.78	0.28	0.12
SFAs[2]	5.10	0.56	5.62	0.91	0.082

SD: standard deviation; Min: minimum; Max: maximum.
[1]∑ other FA: C:12, C17:0, C17:1, C20:1 t, C20:4n6, C23:0, C22:2, C24:0, C20:5n3, C24:1, C22:6n3.

4.2 Phenolic Acids, flavonoids, and saponins content of PL and PLS

The ensiling process increased the concentration of both phenolic acids and flavonoids. A particular content of the main bioactive compounds identified in PL and PLS can be found in Supplementary data, Table 3. PLS contained 47.0 mg/g DM of phenolic acids and 13.0 mg/g DM of flavonoids; before the ensiling process, there was 24.6 mg/g DM of phenolic acids and 3.75 mg/g DM of flavonoids (Table 5). The low

level of saponins in PL and PLS was observed (0.29 mg/g and 3.14×10^{-4} mg/g DM, respectively).

Table 5. Total phenolic acid, flavonoid, and saponin compositions (mg/g dry matter) in Paulownia leaves (PL) and its silage (PLS).

	PL (n = 4)	PLS (n = 4)
Phenolic acids	24.6 ± 0.27	47.0 ± 0.66
Flavonoids	3.75 ± 0.10	13.0 ± 0.31
Saponins	0.29 ± 0.01	$3.14 \times 10^{-4} \pm 0.01 \times 10^{-4}$

4.3 *In vitro* experiment

4.3.1 Batch culture (Experiment 1)

Paulownia affected the rumen fermentation characteristics differently from alfalfa silage. Decrease in pH and methane concentration ($P < 0.01$) were observed in PL and PLS compared to AS (Table 6). In both PL and PLS, methane production (per gram of DM or IVDMD) decreased ($P < 0.01$). However, the greatest decrease was observed in the PLS. The ammonia concentration in PLS decreased compared to PL. The total gas production and total VFA concentrations increased in both experimental groups, but the greatest increase was noted in PL ($P < 0.01$). Concentrations of acetate, propionate, and butyrate acids significantly increased ($P < 0.01$), whereas the concentrations of iso-valerate, valerate, and the acetate-to-propionate ratio significantly decreased ($P < 0.01$) in the PL and PLS groups compared to AS. The C_2/C_3 ratio was lowest in PL ($P < 0.04$). A significant ($P < 0.001$) decrease in *Archaea* was observed in PL and PLS. The lowest *Archaea* population was noted in PLS ($P < 0.01$). The total bacteria and *Holotricha* populations were higher in PL and PLS than in AS. The abundances of *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens*, and *Prevotella* spp. were higher ($P \leq 0.03$) in the PL and PLS than in the AS group.

Table 6. Effects of Paulownia leaves (PL) and their silage (PLS) on rumen fermentation and microbial populations *in vitro* (n = 12).

Item	AS	PL	PLS	SEM	P-value
pH	6.69^{a}	6.54^{b}	6.49^{b}	0.01	<0.01
IVDMD[1]	0.62^{b}	0.66^{a}	0.65^{a}	0.004	<0.01
Ammonia, mM	10.5^{ab}	11.0^{a}	9.77^{b}	0.18	<0.01
Total gas, ml/g DM	283^{c}	302^{a}	296^{b}	1.36	<0.01
CH_4, mmole	0.92^{a}	0.86^{b}	0.84^{b}	0.01	<0.01
CH_4, mmole/g DM	2.31^{a}	2.14^{b}	2.10^{b}	0.02	<0.01
CH_4/total gas, mmole/L	8.17^{a}	7.09^{b}	7.06^{b}	0.09	<0.01
CH_4/IVDMD, mmole/g	3.73^{a}	3.29^{b}	3.17^{c}	0.05	<0.01
Total VFA, mM	35.7^{c}	42.8^{a}	38.4^{b}	0.62	<0.01
Acetate	24.5^{b}	28.3^{a}	25.3^{b}	0.36	<0.01
Propionate	6.95^{c}	9.74^{a}	8.22^{b}	0.18	<0.01
Butyrate	2.83^{c}	3.46^{b}	3.73^{a}	0.06	<0.01
Iso-valerate	0.69^{a}	0.64^{a}	0.54^{b}	0.03	<0.01
Valerate	0.63^{a}	0.59^{b}	0.57^{b}	0.01	<0.01
C_2/C_3 ratio	3.53^{a}	2.91^{b}	3.09^{b}	0.04	0.04
Holotricha, 10^4/mL	0.18^{c}	0.51^{a}	0.24^{b}	0.02	<0.01
Total protozoa, 10^4/mL	2.53	2.56	2.87	0.07	0.09
Archaea, 10^7/mL	1.67^{a}	0.96^{b}	0.68^{c}	0.01	<0.001
Total bacteria, 10^8/mL	7.52^{b}	8.02^{a}	8.21^{a}	0.16	<0.01
*Fibrobactersuccinogenes**	1.00^{c}	2.59^{b}	4.34^{a}	0.44	0.03
*Butyrivibrio fibrisolvens**	1.00^{c}	2.79^{b}	4.82^{a}	0.34	<0.01
Prevotella spp.*	1.00^{c}	3.52^{b}	6.93^{a}	0.59	<0.01

Within each row, means with lower case superscripts (a–c) are significantly different at $P<0.01$; SEM: standard error of the mean.
AS: alfalfa silage; PL: Paulownia leaves; PLS: Paulownia leaves silage; IVDMD: *in vitro* dry matter digestibility; DM: dry matter; CH_4: methane; VFA: volatile fatty acid.
* Relative transcript abundance (ΔΔ CT).

4.3.2 Rusitec (Experiment 2)

Ruminal pH increased linearly and quadratically ($P < 0.01$) with increasing concentrations of PLS with the greatest pH at the highest PLS inclusion (Table 7). The inclusion of PLS in diets increased the total VFA concentration linearly ($P < 0.01$). Molar proportion of acetate to propionate ratio decreased ($P < 0.01$) linearly; however molar proportions of other VFA (except isovalerate) increased linearly with increasing levels of PLS ($P < 0.01$). The degradability of nutrients was not affected by the PLS inclusion in the diets. The total gas production increased ($P < 0.01$) linearly when PLS was included in the diet. Daily methane concentration decreased ($P < 0.05$) linearly, quadratically, and cubically with increasing doses of PLS in diets. Total protozoa and *Ophryoscolecidae* populations decreased linearly ($P < 0.01$) whereas *Isotrichidae* counts increased linearly ($P < 0.05$) with increasing PLS levels. Total archaea, *Methanobacteriales* and *Methanomicrobiales* populations were decreased ($P < 0.01$) linearly by the higher replacement of alfalfa silage with PLS in the diet. The increasing PLS supplementation caused a linear increase of *Streptococcus bovis, Prevotella spp., Butyrivibrio fibrisolvens,* and *Megasphaera elsdenii* ($P \leq 0.05$) abundances. Besides, *M. elsdenii* showed quadratic and cubical responses ($P < 0.05$).

Table 7. The effect of Paulownia leaves silage (PLS) on *in vitro* ruminal fermentation and methane production (n = 4).

Parameters[1]	CON	PLS (g/kg DM)[2]			SEM	Contrast[3]		
		20	40	60		L	Q	C
pH	6.28	6.52	6.62	6.71	0.02	<0.01	<0.01	0.06
Total VFA, mmol/L	63.5	64.9	66.5	69.5	0.48	<0.01	0.19	0.68
C_2/C_3 ratio	3.56	3.19	3.04	2.84	0.05	<0.01	0.18	0.31
CP degradability (g/kg DM)	569	574	578	582	2.74	0.13	0.97	0.93
TGP, mL/d	3722	3881	3958	4151	25.7	<0.01	0.71	0.31
CH_4, mmol/L	9.45	8.10	6.08	5.94	0.21	<0.01	0.03	0.04
Total protozoa, $\times10^3$/mL	14.0	12.3	11.9	10.7	0.25	<0.01	0.62	0.27
Ophryoscolecidae, $\times10^3$/mL	13.2	11.5	10.8	9.17	0.27	<0.01	0.92	0.36
Isotrichidae, $\times10^3$/mL	0.76	0.79	1.15	1.48	0.05	<0.01	0.05	0.27
Total archaea, $\times10^6$/ mL	3.29	2.86	2.55	2.17	0.13	<0.01	0.87	0.84
Methanobacteriales, $\times10^6$/mL	2.46	2.15	1.96	1.68	0.09	<0.01	0.93	0.79
Methanomicrobiales, $\times10^5$/mL	2.40	2.13	2.02	1.66	0.09	<0.01	0.79	0.55
*Streptococcus bovis**	1.06	2.00	5.61	18.85	2.55	<0.01	0.11	0.59
Prevotella spp.*	3.26	6.88	13.78	20.75	2.06	<0.01	0.40	0.28
*Butyrivibrio fibrisolvens**	0.40	0.44	1.52	4.25	1.04	<0.01	0.98	0.52
*Megasphaera elsdenii**	0.73	0.65	3.03	5.03	0.80	<0.01	<0.01	0.02

[1]VFA: volatile fatty acid; DM: dry matter; CP: crude protein; TGP: total gas production;

[2]CON: control diet; PLS: Paulownia leaves silage diet; PLS was used at 20, 40, and 60 g/kg DM of diet replacing alfalfa silage.

[3]L: linear response; Q: quadratic response; C: cubic response. The results are considered to be significantly different at $P \leqslant 0.05$.

4.4 *In vivo* experiments

4.4.1 *In sacco* (Experiment 3)

Parameters of dry matter degradation differed significantly between the AS and both the Paulownia groups (Table 8). The PLS contained the highest soluble fraction ‘a’ and the lowest slowly degradable fraction ‘b’ ($P < 0.001$), whereas the fractional disappearance rate ‘c’ was higher in PL than in the PLS and AS groups ($P < 0.001$). The potential degradability of DM and OM was about 0.05–0.07 higher for PL and PLS than for AS ($P < 0.001$). With regard to OM degradation, the ‘a’ and PD parameters were higher in PLS ($P < 0.001$) than in the PL and AS groups. Besides, PLS showed lowest ‘b’ fraction (0.24) compared to PL and control whereas the fractional disappearance rate ‘c’ did not differ from the DM disappearance ($P < 0.001$). For CP degradation, the PLS was characterized by the highest soluble fraction ‘a’ and lowest fraction ‘b’ ($P < 0.001$) whereas the fractional disappearance rate ‘c’ was significantly higher in PL ($P < 0.001$). The PD was higher in PLS and control than in PL group ($P < 0.001$). Moreover, the ED was the highest in the PLS group and lowest in PL ($P < 0.001$).

Table 8. *In sacco* ruminal degradation kinetics, potential (PD) and effective (ED) degradation of Paulownia leaves (PL) and their silage (PLS) (n = 24).

Parameter	AS	PL	PLS	SEM	P-value
Dry matter degradation					
A	0.51^{c}	0.59^{b}	0.69^{a}	0.01	<0.001
B	0.34^{a}	0.30^{b}	0.21^{c}	0.01	<0.001
C	0.10^{b}	0.30^{a}	0.14^{b}	0.02	<0.001
PD	0.85^{c}	0.89^{b}	0.90^{a}	0.004	<0.001
ED	0.72^{b}	0.84^{a}	0.84^{a}	0.01	<0.001
Organic matter degradation					
A	0.50^{c}	0.55^{b}	0.66^{a}	0.01	<0.001
B	0.33^{a}	0.33^{a}	0.24^{b}	0.01	<0.001
C	0.10^{b}	0.30^{a}	0.15^{b}	0.02	<0.001
PD	0.83^{c}	0.88^{b}	0.90^{a}	0.01	<0.001
ED	0.71^{c}	0.82^{b}	0.81^{a}	0.01	<0.001
Crude protein degradation					
A	0.38^{c}	0.51^{b}	0.65^{a}	0.02	<0.001
B	0.48^{a}	0.20^{b}	0.15^{c}	0.03	<0.001
C	0.11^{c}	0.34^{a}	0.24^{b}	0.02	<0.001
PD	0.86^{a}	0.72^{c}	0.80^{b}	0.01	<0.001
ED	0.69^{c}	0.68^{b}	0.77^{a}	0.01	<0.001

Within each row, means with lower case superscripts (a–c) are significantly different at P <0.01; SEM: standard error of the mean.
AS: alfalfa silage: PL: Paulownia leaves: PLS: Paulownia leaves silage.
a: soluble fraction; b: slowly degradable fraction; c: fractional hourly disappearance rate constant at which b is degraded; PD: potential degradability; ED: effective degradability

4.4.2 The cannulated cows (Experiment 4)

The inclusion of PLS resulted in an increase ($P < 0.01$) in pH and ammonia concentration in the rumen. Besides, pH values were post-feeding time-dependent ($P < 0.01$) whereas ammonia concentration showed treatment × time interaction (Table 9). The inclusion of PLS decreased the molar proportion of acetate ($P < 0.01$), but it increased the molar proportion of propionate (3 h and 6 h after feeding; $P < 0.01$), isovalerate ($P < 0.05$) and valerate ($P < 0.01$). Molar proportions of most individual VFA showed time-dependent variations ($P < 0.05$). The A/P ratio was lower in the PLS group compared to the control group in 3 h and 6 h after morning feeding ($P < 0.01$). Ammonia concentration was affected ($P < 0.01$) by time × treatment interaction, which increased in the PLS diet compared with the control diet ($P < 0.01$) at 3 and 6 h, but not at 0 h. Total *Isotrichidae*, *Dasytricha ruminantium*, *Isotricha prostoma* and *Isotricha intestinalis* populations increased by feeding the PLS diet. The abundances were affected by treatment × time interaction ($P < 0.01$), except for *Isotricha intestinalis*. *Ostracodinium gracile* and *Polyplastron multivesiculatum* ($P < 0.01$) were also affected by treatment × time interaction. The first three protozoa mentioned above were increased by PLS at 3 h and 6 h, but were similar at 0 h; whereas *Ostracodinium gracile* population was greater at 3 h and 6 h, and *P. multivesiculatum* was greater at 0 h, 3 h, and 6 h in the PLS diet than in the control diet. The inclusion of PLS in the diet decreased total protozoa, *Ophryoscolecidae* and *Entodinium* spp. counts ($P < 0.01$).

Feeding PLS to cannulated cows increased ($P < 0.05$) the populations of all bacterial species examined in this study, except *Ruminococcus flavefaciens*, *Ruminococcus albus* and *Lactobacillus* spp. (Table 10). The decrease in abundances of total archaea, *Methanobacteriales* and *Methanomicrobiales* were noted in the experimental group ($P < 0.01$). The inclusion of PLS did not affect degradability of DM, OM, and NDF, but a lower crude protein degradability was observed ($P < 0.01$) in PLS diet. The EE degradability increased due to PLS feeding ($P < 0.05$). Lowered ($P < 0.01$) methane production (g/d) and yield (g/kg DM intake) in PLS diet were noted compared to the CON diet.

Table 9. The effect on replacing alfalfa silage with Paulownia leaves silage (60 g/kg) on ruminal fermentation characteristics measured in rumen-cannulate
(*n* = 4) (Exp. 2).

Variables[1]	0 h[2]		3 h[2]		6 h[2]		Treatment		SEM	*P*-value[4]		
	CON[3]	PLS[3]	CON	PLS	CON	PLS	CON	PLS		T	H	T×H
pH	5.93	6.07	5.92	6.13	6.09	6.18	5.98	6.13	0.02	<0.01	<0.01	0.22
NH_3, mmol/L	8.73^{b}	8.57^{b}	7.84^{b}	11.2^{a}	6.75^{c}	12.4^{a}	7.80	10.7	0.41	<0.01	0.50	<0.01
Total VFA, mmol/L	106	108	106	104	107	107	106	106	0.53	0.64	0.33	0.44
VFA, mol/100 mol												
Acetate	64.5^{a}	64.4^{a}	63.0^{a}	60.2^{b}	63.1^{a}	59.2^{b}	63.5	61.2	0.25	<0.01	<0.01	<0.01
Propionate	22.1^{c}	21.1^{c}	22.4^{c}	24.8^{a}	23.1^{bc}	24.8^{a}	22.6	23.6	0.19	<0.01	<0.01	<0.01
Isobutyrate	0.67	0.75	0.70	0.74	0.65	0.77	0.67	0.75	0.03	0.22	0.96	0.85
Butyrate	9.88^{c}	10.6^{b}	11.1^{a}	10.7^{b}	10.6^{b}	11.8^{a}	10.5	11.0	0.12	0.02	<0.01	<0.01
Isovalerate	1.10^{b}	1.52^{a}	1.13^{b}	1.80^{a}	1.10^{b}	1.84^{a}	1.11	1.72	0.04	<0.01	0.03	0.04
Valerate	1.66^{b}	1.67^{b}	1.66^{b}	1.81^{a}	1.38^{c}	1.86^{a}	1.57	1.78	0.03	<0.01	0.08	<0.01
C_2/C_3 ratio	2.93^{a}	3.06^{a}	2.83^{a}	2.47^{b}	2.75^{a}	2.40^{b}	2.84	2.64	0.03	<0.01	<0.01	<0.01
Microbial populations												
Total protozoa, $\times10^5$/mL	12.1	10.6	13.3	11.3	14.6	11.9	13.2	11.3	0.17	<0.01	<0.01	0.16
Isotrichidae, $\times10^3$/mL	5.64^{c}	6.27^{c}	6.14^{c}	16.8^{b}	6.38^{c}	21.7^{a}	6.05	15.1	0.57	<0.01	<0.01	<0.01
Ophryoscolecidae, $\times10^5$/mL	12.0	10.5	13.2	11.1	14.5	11.7	13.2	11.1	0.17	<0.01	<0.01	0.11
Dasytricha ruminantium, $\times10^3$/mL	5.10^{c}	5.29^{c}	5.21^{c}	15.5^{b}	5.24^{c}	19.8^{a}	5.18	13.6	0.53	<0.01	<0.01	<0.01
Isotricha prostoma, $\times10^3$/mL	0.25^{d}	0.19^{d}	0.49^{c}	0.93^{b}	0.66^{c}	1.37^{a}	0.47	0.87	0.05	<0.01	<0.01	<0.01
Entodinium spp., $\times10^5$/mL	11.9	10.4	13.0	11.1	14.3	11.6	13.1	11.0	0.17	<0.01	<0.01	0.12
*Ostracodinium gracil*e, $\times10^3$/mL	7.16^{c}	5.79^{c}	9.69^{b}	6.64^{c}	12.8^{a}	9.60^{b}	10.0	7.20	0.24	<0.01	<0.01	<0.01
Polyplastron multivesiculatum, 10^3/mL	1.15^{b}	0.70^{c}	2.24^{a}	0.91^{b}	2.45^{a}	1.23^{b}	1.93	0.94	0.07	<0.01	<0.01	<0.01

[1]NH_3: ammonia; VFA: volatile fatty acid.
[2]The ruminal fluid was obtained from each cannulated cow from three locations in the midventral sac of the rumen before morning feeding (0 h), 3 h after morning feeding, and 6 h after morning feeding.
[3]CON: control diet; PLS: Paulownia leaves silage diet.
[4]T: treatment; H: hours.,
[a,b,c,d]Means with different superscript letters differ significantly ($P < 0.05$) among the treatments and hours in a row.

Table 10. The effect of replacing alfalfa silage with Paulownia leaves silage (60 g/kg) on bacteria, methanogens, methane (CH_4) production and digestibility measured in rumen-cannulated cows (n = 4) (Exp. 2)

Item	Treatments[1]		SEM	*P*-value[2]
	CON	PLS		
Microbial populations				
*Fibrobacter succinogenes**	0.13	0.32	0.04	<0.01
*Streptococcus bovis**	1.07	4.61	0.51	<0.01
Prevotella spp.*	3.81	12.91	0.90	<0.01
*Megasphaera elsdenii**	3.98	16.64	1.82	<0.01
Total archaea, $\times 10^8$/ mL	6.28	5.28	0.19	<0.01
Methanobacteriales, $\times 10^8$/mL	4.33	3.44	0.15	<0.01
Methanomicrobiales, $\times 10^7$/mL	3.82	3.21	0.13	<0.01
CP digestibility, g/kg DM	616	584	6.07	<0.01
CH_4, g/d	459	410	9.80	<0.01
CH_4, g/kg DMI	22.1	19.5	0.29	<0.01

[1]CON: control diet; PLS: Paulownia leaves silage diet; the percentage means of how many percentages of alfalfa was replaced with Paulownia silage.
*Abundance (log_{10} number of copies of *rrs* gene/mL of rumen sample).
[2]The results are considered to be significantly different at $P \leq 0.05$.
[3]DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; NDF: neutral detergent fiber.

In the Exp. 4 (cannulated cows), the ruminal fluid of cows fed with PLS was characterized by altered proportions of selected FAs. The level of C10:0, C12:0, C16:0, C16:1, C17:1 and C18:0 decreased ($P < 0.05$; Table 11) whereas the level of C14:1, C:15:0, C:15:1, and C17:0 increased ($P < 0.01$). Regarding the proportion of C8:0, C12:0, C14:1, C:15:0, C:15:1, C16:0, and C16:1, the treatment × time interaction was also observed ($P < 0.05$). The proportion of C18:1 trans-10, C18:1 trans-11, C18:2 cis-9, cis-12, C18:2 cis-9, trans-11, and C18:2 trans-10, cis-12 increased in both treatments ($P < 0.01$) and time-dependent ($P < 0.01$) manners in PLS treatments. The PLS diet tended to decrease C18:1 cis-9 ($P < 0.05$), but increase C18:3n-6 and C18:3 cis-9, cis-12, cis-15 ($P < 0.01$) in treatment-dependent manner. The sum of unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), PUFA, n-6 FA, and the sum of n-3 FA increased ($P < 0.01$) in the experimental group, which resulted in a lower sum of SFA ($P < 0.01$). Time-dependent variation ($P < 0.01$) was observed in all of the above parameters, in addition to the sums from MUFA. The inclusion of PLS increased the sum of trans C18:1, sum of medium-chain FA, n-6/n-3 FA ratio, and PUFA/SFA ratio ($P \leq 0.05$). The treatment × time interaction was noticed for the following FAs: C18:1 trans-10, C18:2 cis-9, cis-12, C18:2 cis-9, trans-11, and C18:2 trans-10, cis-12 and sum of other FA, SFA, UFA, PUFA, n-6 FA, trans C18:1, n-6/n-3 FA ratio, and PUFA/SFA ratio ($P < 0.05$)

Table 11. The effect on replacing alfalfa silage with Paulownia leaves silage (60 g/kg) on fatty acid (FA) proportion (g/100 g of FA) in ruminal fluid (n = 4) (Exp. 2)

Item	0 h		3 h		6 h		Treatment[1]		SEM[2]	*P*-value[3]		
	CON	PLS	CON	PLS	CON	PLS	CON	PLS		T	H	T×H
C10:0	0.13	0.10	0.14	0.10	0.13	0.10	0.13	0.10	0.005	<0.01	0.72	0.98
C12:0	0.44^{a}	0.10^{b}	0.45^{a}	0.10^{b}	0.38^{a}	0.10^{b}	0.42	0.10	0.019	<0.01	0.02	0.04
C14:0	1.12	1.05	1.10	0.96	0.93	0.95	1.05	0.98	0.03	0.04	<0.01	0.12
C14:1	1.18^{b}	1.92^{a}	1.14^{b}	1.89^{a}	1.16^{b}	1.58^{a}	1.16	1.80	0.05	<0.01	<0.01	<0.01
C15:0	1.17^{b}	1.52^{a}	1.10^{b}	1.54^{a}	1.12^{b}	1.44^{a}	1.13	1.50	0.02	<0.01	0.01	0.01
C15:1	0.46^{b}	0.77^{a}	0.47^{b}	0.73^{a}	0.49^{b}	0.66^{a}	0.47	0.73	0.02	<0.01	0.01	<0.01
C16:0	20.4^{a}	19.0^{b}	19.7^{b}	19.5^{b}	20.6^{a}	19.3^{b}	20.3	19.2	0.15	<0.01	0.23	0.02
C16:1	0.48^{a}	0.22^{b}	0.46^{a}	0.22^{b}	0.32^{a}	0.23^{b}	0.42	0.22	0.002	<0.01	0.01	<0.01
C17:0	0.56	0.60	0.52	0.62	0.52	0.59	0.54	0.60	0.009	<0.01	0.08	0.07
C17:1	0.12	0.079	0.10	0.10	0.12	0.087	0.11	0.087	0.005	<0.01	0.95	0.10
C18:0	47.8	45.9	47.4	46.7	46.9	45.1	47.4	45.9	0.42	<0.01	0.17	0.55
C18:1 *trans* 10	1.36^{b}	2.51^{a}	1.23^{b}	2.57^{a}	1.31^{b}	2.78^{a}	1.30	2.62	0.07	<0.01	<0.01	<0.01
C18:1 *trans* 11	0.41	0.67	0.46	0.70	0.46	0.74	0.45	0.70	0.02	<0.01	<0.01	0.36
C18:1 *cis* 9	5.67	5.78	6.06	5.56	6.64	5.96	6.13	5.76	0.14	0.03	<0.01	0.21
C18:2 *cis* 9, *cis* 12	5.27^{c}	7.38^{a}	6.52^{b}	6.78^{b}	6.74^{b}	7.47^{a}	6.14	7.19	0.14	<0.01	<0.01	<0.01
C18:2 *cis* 9, *trans* 11	0.16^{b}	0.86^{a}	0.24^{b}	0.87^{a}	0.21^{b}	0.87^{a}	0.20	0.87	0.03	<0.01	<0.01	0.03
C18:2 *trans 10, cis* 12	0.12^{b}	0.16^{a}	0.15^{b}	0.14	0.15^{b}	0.17^{a}	0.14	0.16	0.005	<0.01	<0.01	<0.01
C18:3 n-6	0.16	0.17	0.16	0.17	0.16	0.19	0.16	0.18	0.005	<0.01	0.21	0.61
C18:3 *cis* 9, *cis* 12, *cis* 15	1.29	1.38	1.15	1.34	1.23	1.34	1.23	1.35	0.02	<0.01	0.01	0.27
Sum of other FA[4]	9.2^{c}	9.84^{a}	9.91^{a}	9.52^{b}	9.58^{b}	10.0^{a}	9.55	9.81	0.12	0.08	0.36	<0.01
Sum of SFA	74.5^{a}	69.4^{b}	73.0^{a}	70.5^{ab}	71.9^{a}	68.7^{b}	73.2	69.5	0.36	<0.01	<0.01	0.02
Sum of UFA	25.5^{c}	30.6^{a}	27.0^{b}	29.5^{ab}	28.1^{b}	31.3^{a}	26.8	30.5	0.36	<0.01	<0.01	0.02
Sum of MUFA	17.3	20.9	17.3	20.4	17.9	20.9	17.5	20.7	0.28	<0.01	0.27	0.65
Sum of PUFA	8.30^{b}	9.73^{a}	9.47^{a}	8.98^{b}	9.56^{a}	10.1^{a}	9.08	9.59	0.15	<0.01	<0.01	<0.01

Sum of n-6 FA	6.47^{c}	8.43^{a}	8.13^{ab}	7.85^{b}	8.33^{a}	8.98^{a}	7.60	8.41	0.17	<0.01	<0.01	<0.01
Sum of n-3 FA	1.29	1.40	1.15	1.32	1.23	1.34	1.23	1.35	0.02	<0.01	<0.01	0.58
Sum of *trans* C18:1	2.38^{b}	4.99^{a}	2.33^{b}	5.03^{a}	2.37^{b}	5.34^{a}	2.36	5.11	0.14	<0.01	<0.01	<0.01
Sum of medium-chain FA	26.2	26.9	25.3	26.8	25.9	26.3	25.9	26.7	0.20	<0.01	0.23	0.09
Sum of long-chain FA	73.6	73.0	74.0	73.0	73.8	73.5	73.8	73.1	0.21	0.01	0.46	0.45
n-6/n-3 FA ratio	5.13^{f}	5.57^{df}	7.80^{a}	6.05^{c}	6.65^{b}	6.69^{b}	6.44	6.09	0.19	0.05	<0.01	<0.01
PUFA/SFA ratio	0.11^{c}	0.14^{a}	0.13^{b}	0.13^{b}	0.13^{b}	0.15^{a}	0.13	0.14	0.003	<0.01	<0.01	<0.01

[1]CON: Control diet; PLS: Paulownia leaves silage diet.
[2]SEM: standard error of means for the main effect.
[3]T: treatment; H: hour.
[4]Other FA include C8, C14:1 *iso*, C14:1 *anteiso*, C15:1 *anteiso*, C16:1 *anteiso*, C17:1 *anteiso*, C18:1 *trans*-6–8, C18:1 *trans* 9, C18:1 *cis* 11, C18:1 *cis* 12, C18:1 *cis* 13, C18:1 *cis* 14, C18:2 *trans* 11, *cis* 15, C20:0, C20:1 *trans*, C21:0, C18:3 *cis* 9, *trans* 11, *cis* 15, C22:0, C23:0, C24:0, and C24:1,
[a,b,c,d]Means with different superscript letters differ significantly ($P < 0.05$) among the treatments and hours in a row.

4.4.3 Commercial dairy cows (Experiment 5)

The inclusion of PLS in the diet of dairy cows affected milk composition and ruminal methane concentration (Table 12). The PLS diets decreased protein and lactose yield ($P < 0.05$), however, it did not affect milk yield, energy-corrected milk (ECM), and fat yield. Replacing alfalfa silage with PLS did not affect the fat content, but the PLS diet decreased protein and lactose content ($P < 0.05$). Milk urea concentration was increased ($P < 0.05$) by PLS feeding. Methane concentration in the exhaled gas decreased ($P < 0.01$) by about 14% in the PLS group compared to the CON group.

The PLS diet increased proportions of C15:0, C16:1, C18:2 cis-9 trans-11, C18:3 cis-9 cis-12 cis-15 and C20:4 n-6 ($P < 0.01$) (Table 13.). The proportions of C18:1 trans-10, C18:1 trans-11, and the sum of trans-C18:1 was decreased ($P < 0.05$) by PLS feeding to dairy cows. The PLS diet decreased the total SFA ($P < 0.05$) proportion, but increased the total UFA ($P < 0.05$) and PUFA ($P < 0.05$) proportions. The PUFA/SFA ratio was higher ($P < 0.05$) in the PLS group than in the CON group, but the n-6/n-3 FA ratio was lower ($P < 0.01$) in the PLS group than in the CON group. Supplementation of PLS increased desaturase indices of C16:1, and rumenic acid / (vaccenic acid + rumenic acid) ($P < 0.05$). Furthermore, the PLS affected the relative transcript abundances of five out of six analyzed genes. Replacing alfalfa silage with PLS resulted in increased mRNA expressions of all genes ($P < 0.01$) except ACACA gene (Fig. 1).

Table 12. The effect of replacing alfalfa silage with Paulownia leaves silage (60 g/kg) on milk production performance and methane concentration of commercial dairy cows (*n* = 16) (Exp. 3)

Item	Treatments[1]		SEM	*P*-value[2]
	CON	PLS		
DM intake	23.5	22.9	0.16	0.11
Milk yield				
Milk, kg/d	33.9	32.5	0.46	0.08
ECM [3], kg/d	35.5	33.8	0.67	0.17
Fat, g/d	1349	1201	68.2	0.23
Protein, g/d	896	831	16.5	0.02
Lactose, g/d	1296	1189	23.5	0.01
Milk composition				
Fat, g/kg	43.8	44.0	0.99	0.91
Protein, g/kg	34.1	33.4	0.16	0.03
Lactose, g/kg	49.3	47.9	0.28	0.01
Urea, mg/L	224	249	3.06	0.02
Methane, μg/L	211	185	7.1	<0.01

[1] CON: control diet; PLS: Paulownia leaves silage diet.

[2]The results are considered to be significantly different at $P \leq 0.05$.

[3]Energy corrected milk calculated according the following equation: ECM = milk yield (kg) × (38.3 × fat (g/kg) + 24.2 × protein (g/kg) + 783.2)/3.140.

Table 13. The effect of replacing alfalfa silage with Paulownia leaves silage (60 g/kg) on milk fatty acids (FA) composition (g/100 g FA) and desaturation (DI) of milk of dairy cows (n = 16) (Exp. 3)

Item[1]	Treatment[2]		SEM	P-value[3]
	CON	PLS		
C15:0	1.46	1.59	0.02	<0.01
C16:1	1.33	1.74	0.05	<0.01
C18:1 *trans* 10	0.53	0.44	0.01	<0.01
C18:1 *trans* 11	0.60	0.51	0.02	0.02
C18:2 *cis* 9, *trans* 11	0.56	0.72	0.01	<0.01
C18:3 *cis* 9, *cis* 12, *cis* 15	0.39	0.47	0.01	<0.01
C20:4 n-6	0.13	0.16	0.002	<0.01
Other FA[3]	4.91	4.73	0.06	0.56
Total FA				
Sum of SFA	67.9	66.2	0.39	0.04
Sum of UFA	32.1	33.8	0.39	0.04
Sum of MUFA	27.4	28.8	0.35	0.05
Sum of PUFA	4.66	4.90	0.05	0.03
Sum of n-3 FA	0.51	0.60	0.01	<0.01
Sum of *trans* C18:1	1.69	1.53	0.02	<0.01
PUFA/SFA ratio	0.07	0.08	0.001	0.02
n-6/n-3 FA ratio	7.89	6.76	0.12	<0.01
DI C14:1/(C14:0+C14:1)[4]	0.28	0.30	0.004	0.01
DI C16:1/(C16:0+C16:1)	0.037	0.051	0.002	0.02
DI RA/(VA+RA)	0.48	0.59	0.01	<0.01

[1]SFA: saturated fatty acids; UFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; MCFA: medium-chain fatty acids; LCFA: long-chain fatty acids: RA: rumenic acid (C18:2 *cis*-9, *trans*-11); VA: vaccenic acid (C18:1 *trans*-11).

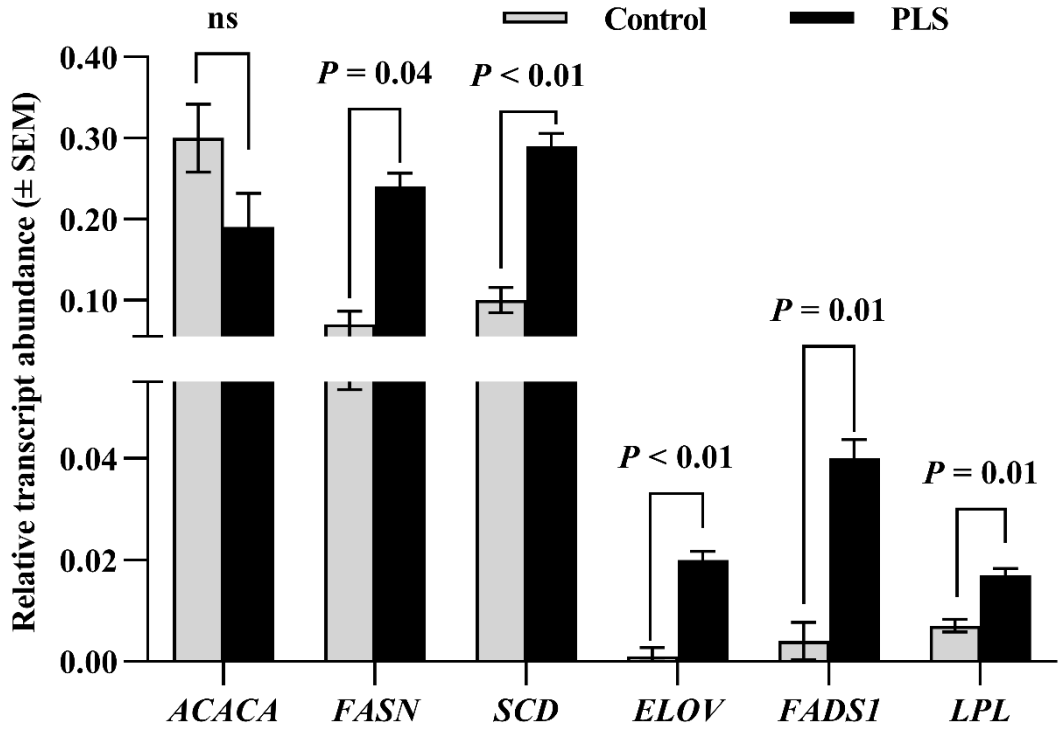
[1]CON: control diet; PLS: Paulownia leaves silage diet.

[2]The results are considered to be significantly different at $P \leq 0.05$.

[3]Other FA include C8, C12, C14 C14:1, C15:1. C16, C17:0, C17:1, C18, C18:1 *trans* 11, C18:1 *cis* 9, C18:1 *trans*-6-8, C18:1 *trans* 9, C18:1 *cis* 11, C18:1 *cis* 12, C18:1 *cis* 13, C18:1 *cis* 14, C18:2 *cis* 9, *cis* 12, C18:2 *trans* 10, *cis* 12, C19:0, C20:0, C20:1 *trans*, C18:3 n-6, C20:5 n-3, C21:0, C20:2, C22:0, C20:3 n-6, C22:1 n-9, C20:3 n-3, C23:0, C22:2, C22:6 n-3 C24:0 and C24:1.

[4]DI C14:1, DI C16:1, DI RA/(VA+RA) were calculated according to Bryszak et al. (2019)

Figure 1. The effect of replacing diet with Paulownia leaves silage (PLS: 60 g/kg) on expression of acetyl-CoA carboxylase 1 (*ACACA*), fatty acid synthase (*FASN*), stearoyl-CoA desaturase (*SCD*), fatty acid elongase (*ELOVL*), fatty acid desaturase 1 (*FADS1*), and lipoprotein lipase (*LPL*) genes in the milk of lactating cows (ns - not significant).



5 Discussion

This Ph.D. thesis is the first complete report on chemical composition and the effects of fresh and ensiled Paulownia leaves on ruminal fermentation, *in situ* nutrient degradation, and methane production in dairy cows. We found that PL and PLS are good sources of nutrients and should be considered as dietary components for ruminants.

5.1 Paulownia leaves characterization of chemical composition

The chemical composition of both PL and PLS showed that Paulownia leaves possess great environmental benefits and good nutritional value so can be utilized as a sustainable dietary ingredient for ruminants. We found that the CP content (averaged 172 g/kg DM) of PL/PLS was comparable to alfalfa silage in terms of the average total CP content (170-190 g CP/kg DM), which is consistent with Ke et al. findings (2015). Compared to Paulownia materials with leaves of selected trees: Populus sp. or Moringa oleifera, the CP content varies widely from 120 g/kg to 300 g/kg (Su and Chen 2020). With our findings, we emphasized that the chemical composition including the CP content of Paulownia leaves depends on the harvest time. Leaves collected in May (as it was collected in the current study) were higher in protein concentration than those collected in the late autumn (Ganchev et al., 2019). Due to this variation, our plant materials' NDF concentration was lower than that reported by Al-Sagheer et al., (2019) and Ganchev et al., (2019). The ensiling contributed to a DM loss of approximately 16%, which is common for silage fermentation (Borreani et al., 2018). The PLS's ammonia content was lower than the good-quality silage, which suggests low AA deamination during ensiling. The biologically active phenolic substances can reduce the production of ammonia (Ke et al., 2015). Besides, Paulownia is a rich source of amino acids. The amino acid profile of the Paulownia leaves is similar to that of alfalfa hay (Al-Sagheer et al., 2019). Despite of proteolysis, our results showed an increase in the relative concentrations of amino acids in ensiled Paulownia, and we attribute this increase to the elevated content of phenolic acids and flavonoids. As a result, this may partially inactivate the proteolytic enzymes in the ensiled materials by forming complexes with proteins (Lee et al., 2008). The formation of ammonia may volatize non-protein N compounds in PLS, potentially increasing the relative concentrations (per 16 g N) of amino acids. Regarding fatty acid composition, the main fatty acid in PL and PLS was C18:3 c9, c12, c15, while the most abundant FAs in PL and PLS were C16:0, C18:1 c9, and C18:2 c9, c12. These

FAs, especially polyunsaturated n-3 acids, resulted in higher content of health-beneficial FAs in the milk. Based on these evidences, we can suggest that Paulownia leaves can be a valuable FA source for ruminants (Alagawany et al., 2022). Furthermore, a higher level of C18:2 c9c12 in PLS may be likely to cause changes in rumen biohydrogenation. Some higher levels of FAs and SFAs in PLS than PL could be due to the loss of VFA, ammonia, and CO_2 (Koivunen et al., 2015). Changes in FA profile in PLS may be also related to microbial activities during the ensiling process. Based on Patra'sPatra (2012) findings, Paulownia leaves are rich in bioactive substances such as triterpenes, phenylpropanoid glycosides, and flavonoids, especially C-geranylated flavonoids which may exert antimicrobial effects. We isolated new compounds from some varieties of Paulownia (Adach et al., 2020), though they are limited to fresh materials. The ensiling in our study increased the concentrations of several bioactive metabolites, in comparison to the fresh leaves. Both PL and PLS are rich in phenolic acids and flavonoids. Specifically, PLS contains a significantly higher content of phenolic acids. We attributed this to the loss of volatile biomass during the ensiling process (Wen et al., 2017). Also, we observed that Paulownia leaves lost around 16% of their DM during ensiling. Another reason for the substantial increase in secondary metabolites in PLS may result from easily accessible metabolites for reagents during the chemical analyses in the ensiled leaves (Szumacher-Strabel et al., 2019). Due to the fact that bindings may be developed in some secondary compounds, they can be more easily extractable by organic solvents in the ensiling process. Additionally, some bioactive components can be converted to other compounds in the processes that occur during ensiling, which are either not present in fresh materials or present in trace amounts. The higher enzymatic activities of lactic acid bacteria during the ensilage process, release bound polyphenols or other bioactive complexes from plant materials. Therefore, they promote the production and accumulation of bioactive metabolites in ensiled materials (Stefanello et al., 2018;Fan et al., 2020).

5.2 Effects of Paulownia on rumen fermentation, microbial modulation of dairy cows in batch culture experiment (Exp.1)

Flavonoids contain various biological properties and may stimulate bacterial growth or structural change in ruminal microorganisms, thus contributing to the digestion of feed in the rumen (Zhan et al., 2017). For example, mulberry leaf flavonoids decrease ruminal populations of methanogens (Ma et al., 2017). But in our study, we noted an increase in total bacterial count, particularly, *F. succinogenes, B. fibrisolvens, and*

Prevotella spp. However, methane production decreased by PL and PLS. This reduction of methane may be attributed to the decreased number of Archaea responding to secondary metabolite activities. As we have observed in the present study, the inhibition of rumen methanogenesis synchronizes with an increase in propionate content. In line with Mitsumori et al., (2012) findings, we also observed high levels of H_2 in the rumen as a result fermentation shifted from acetate to propionate produced by *Prevotella spp*. activity. We also observed an increase in the amount of these bacteria. Besides, elevated propionate along with reduced acetate levels can have negative effects on *F. succinogenes* and *B. fibrisolvens'* activities (Zhang et al., 2020). However, this was not observed in the present experiment. Thus, we can relate the stable acetate content to higher abundances of *F. succinogenes* and *B. fibrisolvens*.

In addition to flavonoids, another secondary metabolite e.g., phenylpropanoid glycosides (PPGs) was found to be dominant in PLS in this study. PPGs express a wide range of biological properties (Uddin et al., 2020). Besides PPGs, acteoside, isoacteoside, and campneoside were other major components of PLS, consistent with findings in Adach et al., (2020) study on Paulownia hybrids. The total phenolic acid content in PLS was as high as 60 mg/g DM, comparable to values reported for the leaves of *Paulownia fortunei* species. That was higher than those in *Paulownia elongata* (He et al., 2016). All these properties increased the nutritional value of both fresh and ensiled Paulownia leaves, as an animal dietary ingredient.

The methanogen activities could be reduced by the acetoside, catalpol, and aucubin. These bioactive compounds in *Plantago lanceolata L.* decreased the H_2 sink in the rumen (Redoy et al., 2020) to reduce methanogen activity. Based on Petrič et al. study (2020), it was shown that dietary substrates containing flavonoids and phenolic acids can potentially reduce methane emission and catalyze promising changes in the rumen environment. We know that certain phenolic acids have toxic effects on ruminal bacteria and protozoa (Berchez et al., 2019), however, no such effects were noted in this study.
On the other hand, researchers reported the inhibitory effects of phenolic acids and polyphenols on methanogenesis accompanied by certain improved fermentation parameters (Bodas et al., 2012). Thus, phenolic acids and flavonoids may directly inhibit the population or activities of methanogens, or both (Bodas et al., 2012). An increase in some VFAs, such as acetate, as observed by Berchez et al., (2019) could be due to the adaptation of ruminal microorganisms with the phenolic acids and flavonoids. This was perhaps the result of the biohydrogenation of phenolic acids from high toxic form to a

less toxic form (Berchez et al., 2019). Our results indicated that PL and PLS can exert a positive effect on ruminal parameters, including a reduction of methane production. This effect makes PL and PLS not only essential sources of nutrients for the rumen but also feed with the potential to reduce the negative impact of animal production on the environment.

5.3 Effects of Paulownia silage in rustic experiment (Exp. 2)

The Paulownia leaves were used as a dietary ingredient in pigs, small ruminants, rabbits, and birds (Al-Sagheer et al., 2019;Alagawany et al., 2022). Recently, we have published promising results of bioactive components from PLS on the ruminal environment (Nowak et al., 2022). Therefore, we are confident to claim that the higher ruminal pH ($P < 0.01$) of PLS diets can be partly explained by BAC activities. Such activities decreased ($P < 0.01$) the acetate-to-propionate ratio by increasing the number of lactate-consuming and propionate-producing bacteria and preventing the ruminal pH reduction (Balcells et al., 2012). We found that although the population of *Streptococcus bovis* increased in the PLS groups ($P < 0.01$), its growth may not increase the production of lactic acid in the ruminal fluid, thus pH reduction (Grazziotin et al., 2020). Increased pH was probably related to the abundance of *Megasphaera elsdenii* ($P < 0.01$) in the PLS diets, as noted both in the present *in vitro* and *in vivo* experiments. *M. elsdenii* utilizes lactic acid as the main source of energy. Thus, it can protect other ruminal microorganisms from the negative impacts of low pH, resulting from lactic acid accumulation (McDonald et al., 1995). High pH positively affects the adhesion of fibrolytic bacteria to the feed particles and their degradation (Sung et al., 2006). As we confirmed in the present study, this phenomenon demonstrated an increase ($P < 0.01$) in the population of some bacteria species such as *Butyrivibrio fibrisolvens* and *Prevotella spp.* in response to PLS supplementation. Zhan et al., (2017) showed that flavonoids (20 to 100 mg/kg body weight) increased the *B. fibrisolvens* population and feed digestion through changes in the population of ruminal microorganisms.

5.4 Effects of Paulownia silage on degradation *in sacco* experiment (Exp. 3)

The ensiling of PL accelerated the degradation of soluble OM and CP fractions and inhibited the degradation of the slowly degradable OM and CP fractions. Higher degradation rates are most often associated with higher levels of NFC in feeds, which

contain less fiber and are easy to ferment (Zhao et al., 2016). The NFC content in PL and PLS was 345 g/kg DM and 333 g/kg DM, respectively. Similar starch levels were found in AS and PLS (15 to 12 g/kg DM, respectively). According to Zheng et al., (2016), it is rather the presence of polysaccharide polyols that determines higher degradation. Polysaccharide polyols are also present in the leaves of other plants (Zheng et al., 2016). Sugar alcohols may increase microbial activities, degrading the substrate to higher propionic acid content (Biebl et al., 2000); this was also observed in the present *in vitro* study. Fernández et al., (2019) also showed greater digestibility of the OM and CP of orange leaves than that of dried alfalfa. As indicated in this study, leaves can be a good alternative to roughage. The greater 'b' fraction in DM degradability in AS could be explained by the high CP content (215 g CP/kg DM). We found that the degradability values of AS, PL, and PLS were affected by saponins, phenolic acids, and flavonoids. Existing literature didn't show their exact mode of action, however, there is a general assumption that the energy value of the carbohydrate digested in the intestine is higher than that of the carbohydrate digested in the rumen. On the other hand, more energy is provided to ruminants by rumen-digested carbohydrates Zhao et al., (2016). As ruminants for microbial protein synthesis relies on ruminal carbohydrate digestion for their main source of energy. So PLS could serve as a promising dietary component for ruminants. Additionally, there is an increase in the CP degradability during the ensilage process, which may indicate an effect of ensiling on the availability of protein (including AA) to rumen microorganisms. Therefore, feeding PL or PLS to ruminants with greater amounts of easily fermentable nonstructural carbohydrates (NFC) should be added to the diet to synchronise protein and energy balance in the rumen.

5.5 Effects of Paulownia silage on cannulated cows (Exp. 4)

In the present experiment, we supplemented the cannulated cows with flavonoids at the dose of 29 mg/kg of body weight, corresponding to a lower dose than in the study by (Zhan et al., 2017). Besides changes in pH, the PLS diet increased the ammonia concentration ($P < 0.01$). We explained this by higher ruminal CP degradation than the control with non-synchronization of available energy and ammonia in the ruminal fluid (Brooks et al., 2014). On the other hand, the total-tract degradability of CP in the PLS-fed cows was reduced ($P < 0.01$). Such differences may be the result of different degradation of protein or distinct solubility of feed protein from alfalfa and Paulownia

leaves silages (Huang et al., 2021). Changes in the observed ammonia concentration may also be associated with changes in the number of protozoa and bacteria. Even though ammonia concentration can be decreased due to reduced recycling of engulfed bacterial protein by protozoa, the decrease in the protozoal number may increase the bacterial activities or growths in the rumen. The *Ophryoscolecidae* protozoa decreased ($P < 0.01$), whereas *Isotrichidae* protozoa significantly increased ($P < 0.01$) in the PLS diet. Based on a prior study, *Ophryoscolecidae* protozoa have a higher bacterivory activity (Ayemele et al., 2021), which explains that a decrease in the number of *Ophryoscolecidae* could increase the selected bacterial populations. In the current study, number of proteolytic bacteria such as *Prevotella spp*. ($P < 0.01$) and *B. fibrisolvens* ($P < 0.05$) in PLS diets increased considerably, both *in vitro* and *in vivo*. *Prevotella spp.* in the rumen are the predominant proteolytic bacteria with a diverse and broad range of peptidase activities which represent 20% to 60% of the bacterial abundance (Patra and Yu 2022). Therefore, the PLS diet may increase the proteolytic activities in the rumen, resulting in higher ammonia concentration in the rumen.

The inhibition of ruminal methanogenesis is usually related to an increase in propionate concentration due to competition for hydrogen. High levels of metabolic hydrogen in the rumen may shift fermentation towards propionate production by *Prevotella spp*. Which were increased ($P < 0.01$) in the PLS diets. Ruminal *Prevotella* species use different pathways for propionate production by utilizing metabolic hydrogen via succinate or acrylate pathways for fermentation of sugars and lactate (Mitsumori et al., 2012). Moreover, as confirmed in the present study, elevated propionate can result from flavonoid supplementation (0.2 mg/g DM), which reduced methane production ($P < 0.01$) and population of methanogenic archaea ($P < 0.01$), as well as increased ($P < 0.01$) the population of *M. elsdenii* (Seradj et al., 2014).

Consistent with findings from our previous *in vitro* study, we found that Paulownia leaves with high total polyphenols content (31 or 35 g/kg DM) lowered the ruminal methane production and the methanogen populations without affecting substrate degradability and volatile fatty acid concentrations (Puchalska et al., 2021). Besides, the addition of flavonoid-rich plants may negatively affect the growth of some protozoa-associated methanogens (Patra and Saxena 2010). As estimated, protozoa-associated methanogens are responsible for up to 37% of methane production (Newbold et al., 2015). In the present study, results obtained from rumen-cannulated dairy cows confirm those from the *in vitro* experiment, including reduced methane emissions ($P < 0.01$), increased

pH ($P < 0.01$), and ammonia concentration ($P < 0.01$), elevated propionate and butyrate concentrations ($P < 0.05$), decreased methanogens ($P < 0.01$) and *Ophryoscolecidae* numbers ($P < 0.01$), and increased *Isotrichidae* population ($P < 0.01$). Variations of protozoa behavior in the ruminal fluid from rumen-cannulated cows may suggest their distinct reaction to the BAC of PLS origin.

Another effect of BAC x microorganisms' interaction is the increased ($P < 0.01$) population of *Fibrobacter succinogenes*. The increase in *F. succinogenes* and *B. fibrisolvens* populations might be a result of the presence of phenolic acids in PLS. We found that some phenolic acids such as hydroxycinnamic acid, syringic acid, and p-hydroxybenzoic acid, stimulate specific bacterial populations in a dose-dependent manner (Borneman et al., 1986). Though high concentrations of polyphenols are toxic to ruminal bacteria, a low concentration of polyphenol extract was also observed in this study that stimulates the *F. succinogenes* in the rumen (Singh et al., 2022). Elevated numbers of *F. succinogenes* known as non-H_2 producers may mitigate methane production through H_2 restriction (Mitsumori et al., 2012). In addition, increased propionate and less acetate reduced the abundance of *F. succinogenes* and *B. fibrisolvens* (Zhang et al., 2020). We did not observe such relations regarding *F. succinogenes* and *B. fibrisolvens* population but we noticed a decrease in acetate ($P < 0.01$) and an increase in propionate concentration ($P < 0.01$) in the ruminal fluid.

5.6 Effects of Paulownia silage on commercial dairy cows (Exp. 5)

In this study, we observed a reduction in milk production, protein, and lactose yield ($P < 0.05$) in response to PLS treatment. As Khorsandi et al. (2019) reported, milk protein and lactose yield reduced after using 120 g/kg DM of pomegranate by-product silage, rich in phenolic compounds (49 g/kg DM) which were also noted in our study. The inclusion of forages rich in phenolic compounds can decrease CP digestibility, as was observed in the current experiments. Moreover, in our previous study, the rapidly soluble fraction of protein degradation was higher in PLS than in alfalfa silage. This difference indicates an imbalance of available energy resulting in higher ammonia concentration in the ruminal fluid (Huang et al., 2021). Higher ammonia levels in the ruminal fluid may interact with milk parameters which results in higher urea content ($P < 0.05$) in milk when the diet was supplemented with PLE (Nousiainen et al., 2004). In this context, it may be

important to balance the dairy cow rations for easily fermentable nonstructural carbohydrates such as concentrates.

Regarding the fatty acids, an increase in C15:0 proportion ($P < 0.01$) in the ruminal fluid and milk suggests higher activities of *F. succinogenes*. These microorganisms are responsible for the synthesis of some FAs, including odd-chain (mainly C15:0) and branched-chain FAs (Zhan et al., 2017). The PLS diet also modulated the biohydrogenation of long-chain FA such as C18:2 cis-9, cis-12. According to Szczechowiak et al., (2016), BAC derived from lingonberry (*Vaccinium vitis-idaea*) affected the biohydrogenation process with an increase in conjugated linoleic acid isomer (CLA isomer; C18:2 cis-9, trans-11) and vaccenic acid (C18:1 trans-11) as intermediates, and these findings were consistent with our study. In the case of two FAs, markers of the biohydrogenation process such as C18:1 trans-11 and C18:2 cis-9, trans-11, parallel changes were noticed in ruminal fluid and in milk. Synthesis of these two isomers is dependent on the activity of *B. fibrisolvens*, one of the dominant group A bacteria responsible for transforming UFA into SFA during ruminal biohydrogenation (Singh et al., 2022). Despite an increase in the abundance of *B. fibrisolvens* in the ruminal fluid of the PLS group, we did not notice an elevation in SFA in favor of UFA. This may be due to the activity of Δ9-desaturase affecting the proportion of the C18:1 trans-11 (Bryszak et al., 2019). On the other hand, BAC may reduce Δ9-desaturase activity, which may reduce the content of C18:1 trans-11 in the milk (Henke et al., 2017). Another factor influencing the concentration of the C18:1 trans-11 is the activity of the *B. proteoclasticus*, the B bacteria group being capable of efficiently biohydrogenation PUFA to SFA (Huws et al., 2011). *B. proteoclasticus* is recognized as an important bacteria that converts C18:1 trans-11 to C18:0, meanwhile most sensitive to changes in dietary PUFA (Van de Vossenberg and Joblin 2003;Wallace et al., 2006). Despite an increase in the population of *B. proteoclasticus* in response to PLS, the proportion of C18:1 trans 11 did not reduce. The increased PUFA content was also unrelated to this bacterial species' reduction.

Milk quality in terms of FA composition also depends on the activity of gene expression in the mammary gland. We investigated the transcript expression of six genes controlling FA metabolism. The relative transcript abundance of 5 genes increased in response to PLS. The fatty acid synthase (FASN), a multifunctional protein, is responsible for *de novo* synthesis of long-chain PUFAs (Ye 2021). An increase in the FASN transcript level was not accompanied by an elevation in long-chain FAs although the total and the n-3 PUFA content increased, which improves milk quality. Another factor affecting the

elevation of the PUFAs may be the higher transcript level of the ELOVL5 gene. This gene encodes a protein that is responsible for extending long-chain PUFA (Toral et al., 2013;Zhu et al., 2017). According to researchers, the increase in mRNA level of ELOVL5 gene elevated both n-6 and n-3 PUFA contents in milk. We correlated the lack of the ELOVL5 effect on the n-6 PUFA to higher transcript abundance of FADS1, a gene limiting the n-3/n-6 rate of PUFA synthesis (Ibeagha-Awemu et al., 2014). Our study observed a major increase for n-3 FAs (e.g., C18:3 cis-9, cis-12, cis-15) whereas the proportion of only C20:4 n-6 increased without changing the total n-6 PUFA. We did not observe a limitation of the C18:2 trans-10, cis-12 proportion, except for the increase in the abundance of the LPL mRNA. Unlike the present study, Bryszak et al. (2014) described the decreased level of the LPL transcript with the reduced proportion of this FA. Milk quality is also influenced by MUFA content, especially C18:1 cis-9, which is influenced by the SCD gene-regulating *de novo* synthesis of endogenous FAs (Garnsworthy et al., 2010). Despite a higher transcript content of SCD gene in the PLS group, only an increasing trend of C18:1 cis-9 proportion was observed for PLS diet.

Summary

Paulownia leaves in fresh (PL) and ensiled forms (PLS) are rich sources of CP with a high proportion of essential AA. Both forms contain considerable amounts of phenolic acids and flavonoids. These bioactive compounds improved the rumen fermentation characteristics and reduced the methanogen counts which resulted in lower methane production in both *in vitro* and *in vivo* studies. This might be also due to other interactions among bioactive components with ruminal microbiota, which suggest a more multidirectional mode of action of the used amount and type of Paulownia leaves.

Dietary PLS (60 g/kg DM), which represented 65 g/d/cow of phenolic acids and 18 g/d/cow of flavonoids, reduced the methanogenesis and beneficially modulated the ruminal fermentation and biohydrogenation processes without any negative impact on milk production and the performance of lactating dairy cows. Simultaneously, improved milk FA profile including greater proportions of total UFA, PUFA, conjugated linoleic acid, and C18:1 trans-11 along with a reduction of n6/n3 fatty acids ratio.

In short, feeding forage prepared from Paulownia leaves (60 g/kg DM) demonstrated the beneficial effects on dairy cows. However, better energy and protein synergy is needed for feeding this diet.

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Statement no. 1

We hereby declare the contribution of **Mr. Huang Haihao** in preparing the manuscript: *Chemical and phytochemical composition, in vitro ruminal fermentation, methane production, and nutrient degradability of fresh and ensiled Paulownia hybrid leaves* by: **Huang Haihao**, Malgorzata Szumacher-Strabel, Amlan Kumar Patra, Sylwester Ślusarczyk, Dorota Lechniak, Mina Vazirigohar, Zora Varadyova, Martyna Kozłowska, and Adam Cieślak in the Animal Feed Science and Technology 279 (2021): 115038, as follows:

1- Introducing the idea of the manuscript
2- Participation in the creation of the research hypothesis
3- Collecting and preparing fresh and ensiled Paulownia leaves. Conducting the *in vitro*, *in sacco,* and *in vivo* experiments performing the basic chemical analysis, determination of ruminal pH, concentration of ruminal ammonia and volatile fatty acids, ruminal total gas production and ruminal methane concentration analysis, determining the number of ruminal protozoa, analysis of the fatty acids profile of milk, feed, and ruminal samples.
4- Contributing to drawing the ideas of discussing the results.
5- First write the original draft.
6- Replying to the journal reviewer's concerns in round 1.

And based on his contribution we can surely confirm his contribution as 55% of the overall work.

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Statement no. 2

We hereby declare the contribution of **Mr. Huang Haihao** in preparing the manuscript: *The effect of ensiled Paulownia leaves in a high-forage diet on ruminal fermentation, methane production, fatty acid composition, and milk production performance of dairy cows* by: **Huang Haihao**, Dorota Lechniak, Malgorzata Szumacher-Strabel, Amlan Kumar Patra, Martyna Kozłowska, Pawel Kolodziejski, Min Gao, Sylwester Ślusarczyk, Daniel Petrič, and Adam Cieslak in the Journal of Animal Science and Biotechnology 13, no. 1 (2022): 1-19, as follows:

1- Introducing the idea of the manuscript
2- Participation in the creation of the research hypothesis
3- Conducting the *in vitro* and *in vivo* experiments performing the basic chemical analysis, determination of ruminal pH, concentration of ruminal ammonia and volatile fatty acids, ruminal total gas production and ruminal methane concentration analysis, determining the number of ruminal protozoa, analysis of the fatty acids profile of milk, feed, and ruminal samples.
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Chemical and phytochemical composition, in vitro ruminal fermentation, methane production, and nutrient degradability of fresh and ensiled *Paulownia* hybrid leaves

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ABSTRACT

Paulownia is a genus of fast-growing trees that generate a huge mass of leaves, which can be utilized as a feed resource for ruminants. In the present study, the chemical and phytochemical composition, in vitro ruminal fermentation and methane production, and nutrient degradability of fresh *Paulownia* hybrid leaves (PL) and its silage (PLS) were investigated. The crude protein content of PL and PLS ranged from 132 to 199 g/kg dry matter, which was comparable to alfalfa silage (AS). Ensiling of PL increased the amount of both phenolic acids and flavonoids. The amino acid content increased, and total saturated fatty acid concentration tended to increase in PLS compared with PL. In the in vitro study with the ruminal fluid (batch culture), PL and PLS decreased pH ($P < 0.01$) and methane emission compared with AS. Total archaea counts were lowest in PLS, intermediate in PL, and highest in AS. *Fibrobacter succinogenes, Butyrivibrio fibrisolvens,* and *Prevotella* spp. were higher ($P \leq 0.03$) in the PL and PLS than the AS group. Ruminal ammonia concentration was lower ($P < 0.01$) in PLS than in PL. The total gas production and total volatile fatty acid concentrations were higher ($P < 0.01$) in both PL and PLS than in AS. The concentrations of acetate in PL and of propionate in PL and PLS were greater than those in AS. In the *in sacco* experiment, the potential degradability of DM was higher ($P < 0.01$) for PL and PLS

Abbreviations: AS, alfalfa silage; PL, fresh *Paulownia* leaves; PLS, ensiled *Paulownia* leaf; DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; NFC, nonfiber carbohydrates; NDF (aNDFom), NDF assayed with a heat stable amylase and expressed exclusive of residual ash; ADF (ADFom), ADF expressed exclusive of residual ash; GE, gross energy; SPE, solid phase extraction; UHRMS, ultra-high-resolution mass spectrometry; FAME, fatty acid methyl esters; CLA, conjugated linoleic acids; GC, gas chromatograph; IVDMD, in vitro dry matter digestibility; FISH, fluorescence in situ hybridization; PD, potential degradability; ED, effective rumen degradability; AA, amino acids; EAA, essential amino acids; NAA, nonessential amino acids; FA, fatty acids; SFA, saturated fatty acids; UFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; VFA, volatile fatty acids; PPGs, phenylpropanoid glycosides.

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than for AS. PLS had significantly the greatest potential degradability and effective degradability compared to the other groups ($P < 0.001$). It can be concluded that PL and PLS can mitigate methane production by inhibiting methanogens and improving ruminal fermentation characteristics. PL and PLS may serve as valuable dietary components for lactating dairy cows, which can be used year-round on a large scale; however, these in vitro results need to be validated under in vivo conditions.

1. Introduction

Population growth leads to increased demand of foods including ruminant origins, which in turn can negatively affect the environment. There is need to expand alternative plant feed resources to effectively feed ruminants by meeting their nutritional requirements while simultaneously reducing environmental burdens, such as mitigation of greenhouse gas emissions. The ideal source of plant feeds should have a rapid growth rate, resistance to extreme climate conditions, and high nutritional value. Considering the large amount of forage required by a dairy cow, identifying a source of utilizable plant by-products that could supplement the diets is of key importance, and has the potential to reduce both the production costs and the environmental pollution. The group of plants that may meet these requirements is *Paulownia* (family *Paulowniaceae*) from China and East Asia (García-Morote et al., 2014).

Several species (6–17) have been recognized in the genus *Paulownia* (*P.*), of which the most important species are *P. albiphloea*, *P. australis*, *P. elongata*, *P. fargesii*, *P. fortunei*, *P. catalpifolia*, *P. kawakamii*, and *P. tomentosa* (Yadav et al., 2013; Bodnár et al., 2014). These plants are known for their rapid growth rate and very intense photosynthesis resulting from a type C4 cycle and large leaves with diameter up to 90 cm (Icka et al., 2016). The tree trunk is used in the timber industry, thus commercial production of *Paulownia* trees generates a huge mass of leaves commonly used as natural fertilizer. At the age of 8–10 years, the leaf mass of a *Paulownia* tree is about 100 kg. When combined with a density of 540 trees/hectare, *Paulownia* can produce an impressive amount of green mass (Bodnár et al., 2014). Moreover, tree leaves can be pruned to obtain huge amount forage materials for feeding of ruminants, when the nutritive values of the leaves are very high. The nutritional value of *Paulownia* leaves, particularly of *P. elongata*, was investigated in several studies (Bodnár et al., 2014; Al-Sagheer et al., 2019). However, the detailed characteristics of *Paulownia* leaves with respect to biologically active substances and effect on ruminal fermentation, including methane production have not yet been published. Our preliminary study revealed that high levels of crude protein (CP) and bioactive components were present in fresh and ensiled leaves harvested from *P. tomentosa* × *P. fortunei* hybrid trees in the month of May (unpublished data). Woody forage plant species and their bioactive substances modulated ruminal microbial fermentation including mitigation of methane production (Bodas et al., 2012). We hypothesized that *Paulownia* leaves with high concentrations of CP and bioactive components may modulate ruminal fermentation and nutrient degradation, and thus can be considered valuable dietary components for dairy cows. The objectives of this study were to determine the chemical and phytochemical composition of fresh and ensiled leaves of the *P. tomentosa* × *P. fortune* hybrid tree and to investigate their effects on in vitro rumen fermentation characteristics and in situ nutrient degradation.

2. Material and methods

The study was approved by the Local Ethical Commission for Animal Research (permission no. 14/2019). All the experimental procedures were conducted following the guidelines of the National Ethical Commission for Animal Research (Ministry of Science and Higher Education, Poland).

2.1. Plant material and ensiling

The fresh (PL) and ensiled (PLS) *Paulownia* leaves used in this study were obtained from *Paulownia tomentosa* × *Paulownia fortunei* hybrid tress. The leaves were collected over two consecutive years (2018 and 2019) from two-year-old and three-year-old plantations. In each year, four Polish plantations located near Grzybno (heavy loamy soil, class IV, 1000 trees), Modrze (light permeable soil, class IV, 386 trees), Gierłatowo (sandy soil, class III, 400 trees), and Chorzyna (sandy lands, class IV, 1200 trees) were used. Eight (four/year × 2 years) representative samples of *Paulownia* leaves were collected at the end of May when the trees were shaped. Leaf samples from at least fifty trees of each plantation were collected, analyzed as fresh and ensiled, and then lyophilized.

Paulownia leaves from each of the 4 plantations were ensiled within 8 h after harvesting with the biological additive Agricol Sil (Microferm, UK) in three plastic microsilos (three replicates per plantation per year), each consisting of plastic drums of 4 dm^3, 15 cm in diameter and 49 cm height. Thus, 12 microsilos were ensiled in each year with a total of 24 microsilos over two years. The same ensiling protocol was used each year according to Szumacher-Strabel et al. (2019). Approximately 2.4 kg of chopped fresh PL was immediately packed into microsilos, sealed with two screw tops (internal and external) and stored at ambient temperature (20–25 °C) for 8 weeks. After the ensiling period, the microsilos were opened, and samples were obtained for chemical analysis. Samples (about half part) of the ensiled materials were lyophilized using a freeze-drier (Christ Gamma 2–16 LSC, Martin Christ, Osterode am Harz, Germany) and kept in dark for phenolic acid and flavonoid analysis, and for in vitro and in situ experiments. The alfalfa silage (AS) was also used as a control for the in vitro and in situ experiments. The alfalfa was harvested at about the 10 % bloom stage from a three-year-old plantation located near Września (clay lands, class III) and 4 samples from 4 different spots of the plantation were collected. Before ensiling, alfalfa was wilted to 300 g/kg DM content and chopped to approximately 1.5 cm particle length. Like PL

ensiling, the same biological additive in the same dosage was applied and prepared for AS.

Before the in vitro (batch culture) and in situ (rumen *in sacco*) experiments, all the *Paulownia* samples were pooled plantation area-wise and thoroughly mixed (separately for PL and PLS). One representative and homogeneous sample was prepared for each plantation. Finally, 4 samples of PL and 4 samples of PLS were prepared. One sample represented one plantation. Regarding AS silages, one representative and homogeneous sample from each area was prepared. Finally, 4 AS samples were tested.

2.2. Experimental design and management

2.2.1. Batch culture fermentation experiment

The whole procedure for preparing and running a short-term batch culture fermentation was carried out following the modified procedure described by Cieslak et al. (2016). The rumen inoculum was obtained from three ruminal cannulated Polish Holstein-Friesian dairy cows (body weight 625 ± 25 kg, second month of lactation) before the morning feeding. The rumen inoculum donors were fed 24 kg dry matter (DM) of a total mixed ration (TMR), composed of maize silage (352 g/kg DM), alfalfa silage (172 g/kg DM), grass silage (75 g/kg DM), beet pulp (101 g/kg DM), brewer's grain (63 g/kg DM), extracted rapeseed meal (38 g/kg DM), concentrate (189 g/kg DM), and mineral-vitamin mixture (10 g/kg DM). The PL and PLS were included as sole substrate (400 mg in DM). Alfalfa silage (400 mg in DM) was used as a control to compare with tested feeds (PL and PLS) because of its similar nutritive value to PL (Table 1) and AS is a commonly used feed for ruminants. In the vitro study, PL and PLS were used as only substrate in the fermentation media to rule out other factors related to the diets and to obtain ruminal fermentation responses of PL and PLS in comparison to AS used as only substrate. The experiment was repeated for three consecutive days. Twelve *Paulownia* (PL, PLS) samples (4 plantations × 3 independent runs) and 4 AS samples (4 areas x 3 independent runs) were analyzed. A short-termin in vitro batch culture system was used to determine the basic rumen fermentation, methane production, and microbial populations.

2.2.2. In sacco experiment

Three lactating multiparous Polish Holstein Friesian cows (625 ± 25 kg body weight) fitted with rumen cannulas (10 cm, Bar Diamond, Parma, Idaho, USA) were used. The cows were fed twice daily at 06:00 and 18:00 h, the TMR consisting of 400 g/kg of alfalfa forage, 300 g/kg of grass forage, and 300 g/kg of concentrate (wheat grain, extracted rapeseed meal, extracted soybean meal, and mineral and vitamin premix). Clean water was available ad libitum. The cows were adapted to the diet for three weeks before the in-situ incubation was started. PL and PLS were tested and AS was used as a control to compare the tested feeds for their degradability. All substrates were used as lyophilized materials. Samples of 2.75 g DM were placed in each Dacron bag (6 × 13 cm; pore size 50 μm) and sealed with insoluble surgical sutures approximately 1 cm below the upper edge. They were then placed in a large mesh (35 cm × 60 cm) with fine sieves (0.5 cm × 0.5 cm) that permit the ruminal fluid to freely access the feed samples placed in the bag. Weights were used for better placement of the mesh with the incubated bags in the rumen content. Bags were incubated in the rumen of each cow for 0, 2, 4, 8, 12, 24, 48 and 72 h. The zero-hour bags were not placed in the rumen but were treated with distilled water at 39 °C for 15 min. Four bags per tested feed at 0, 2, 4, 8, 12, and 24 h, and eight bags for 48 h and 72 h with a total of 40 bags were used in the rumen of each cow. For each incubated feed (AS, PL, or PLS), three cows were used, and the procedure was repeated twice. In total, 240 bags (40 bags × 3 cows × 2 repetitions) were incubated for each feed (24 replicates per PL or PLS, i.e., 3 cows × 4 plantations × 2 runs and 24 replicates per AS, i.e., 3 cows × 4 areas × 2 runs). After the appropriate incubation time, the bags were removed from

Table 1
Chemical composition of alfalfa silage (AS; n = 4), *Paulownia* leaves (PL; n = 8), and *Paulownia* leaf silage (PLS; n = 24).

	AS		PL				PLS				
	Mean	SD	Mean	SD	Min	Max	Mean	SD	Min	Max	P-value
Dry matter (g/kg as feed)	292	1.96	275	43.0	181	330	232	21.1	217	275	0.14
Organic matter (g/kg DM)	870	4.48	886	11.7	840	901	875	16.5	859	899	0.10
pH	4.56	0.04	—	—	—	—	4.81	0.35	4.41	5.34	—
NH_3-N (g/kg TN)	80.9	5.81	—	—	—	—	28.1	9.53	19.0	56.2	—
Lactic acid (g/kg DM)	35.6	3.17	—	—	—	—	20.0	7.88	16.1	28.9	—
Acetic acid (g/kg DM)	19.0	5.12	—	—	—	—	7.29	1.51	4.69	9.54	—
Propionic acid (g/kg DM)	1.12	0.34	—	—	—	—	1.31	0.62	0.50	2.27	—
Butyric acid (g/kg DM)	3.05	1.58	—	—	—	—	1.17	0.33	0.56	1.79	—
Nutrients DM (g/kg DM)											
Ash	121	4.38	114	11.7	99.1	141	123	17.7	93.0	160	0.10
Crude protein	215	3.81	175	25.7	132	199	170	10.4	145	192	0.50
Crude fat	22.3	1.09	24.4	3.45	11.7	30.4	27.8	9.33	13.1	42.3	0.25
ADFom	227	8.82	295	53.8	240	442	333	42.9	275	411	0.19
aNDFom	368	12.2	341	58.4	279	436	350	45	284	477	0.52
NFC	279	13.5	345	80.5	241	443	333	83.7	247	394	0.59
Starch	15	3.98	19.6	6.98	6.38	30.4	12.1	3.99	8.60	19.3	0.006
Gross energy, MJ/kg DM	15.5	0.54	14.2	0.76	12.8	16.0	13.2	0.81	11.3	14.2	0.004

DM: dry matter; TN: total nitrogen; aNDFom: neutral detergent fiber without ash and assayed with α-amylase; ADFom: acid detergent fiber without ash; NFC: nonfiber carbohydrate: Min: minimum; Max: maximum; SD: standard deviation.

the rumen and the fermentation of the material in the bags was inhibited by placing the bag in cold water. The bags were then rinsed with cold water until clean water was obtained. The bags were then stored in a freezer at −20 °C until ready to be dried. The bags were dried at 60 °C for 48 h using a dryer with forced air circulation. The dried materials were then weighed to obtain the mass of materials after incubation. After weighing, the samples were ground to pass through a 1 mm sieve using a ZM 200 mill (Retsch, Düsseldorf, Germany). The potential degradation (PD) and effective rumen degradability (ED) of DM, organic matter (OM), and CP were calculated as described below.

2.3. Sample analysis

2.3.1. Chemical analysis

Samples of AS, PL and PLS were analyzed following AOAC methods (Horwitz and Latimer, 2007) for dry matter (DM, method no. 934.01) and ash (method no. 942.05). Crude protein (method no. 976.05) content was determined using a Kjel-Foss Automatic 16210 analyzer (Foss Electric, Hillerød, Denmark) and ether extract (EE, method no. 973.18) with a Soxhlet System HT analyzer (FOSS, Hillerød, Denmark). The OM content was calculated from the differences between DM and ash contents. The concentrations of neutral detergent fiber (NDF) and acid detergent fiber (ADF) in feed samples were determined as described by Van Soest et al. (1991), including amylase and sodium sulfite; they were therefore expressed without residual ash (aNDFom and ADFom).

Gross energy (GE) content was determined using an adiabatic bomb calorimeter (KL 12 Mn, Precyzja-Bit PPHU, Bydgoszcz, Poland). Starch concentration was determined by the polarimetric method, following Polish Standard PN-R-64785:1994, and using a polarimeter POL-S2 (Polygen, Wrocław, Poland). The concentration of nonfiber carbohydrates (NFC) was calculated using the formula: NFC, g/kg DM = 1000 - (CP, g/kg DM + EE, g/kg DM + Ash, g/kg DM + NDF, g/kg DM).

2.3.2. Analysis of phenolic acids, flavonoids, and saponins

Plant samples (PL and PLS) preparation and extraction were caried out as described previously (Petrič et al., 2020). All the analyses were performed in duplicate for 4 samples (plantation areas) stored in a freezer at −20 °C before analysis.

Paulownia bioactive compounds (polyphenols and saponins) were analyzed by ultra-high-resolution mass spectrometry (UHRMS) on a DionexUltiMate 3000RS system (Thermo Scientific, Darmstadt, Germany) with a charged aerosol detector interfaced with a high-resolution quadrupole time-of-flight mass spectrometer (HR/Q-TOF/MS, Compact, Bruker Daltonik, Bremen, Germany). The protocols of chromatographic separation, operating parameters, flow rate, spectra, collision energy, and spectra processing for determination of polyphenols in PL and PLS were followed according to Petrič et al. (2020). Chromatographic separation, operating parameters, collision energy, and spectra processing of determination of saponins were performed in line with a previously published protocol (Szumacher-Strabel et al., 2019) using a BEH C18 column (2.1 × 100 mm, 1.7 μm, Waters). All the analyses were performed in triplicate.

2.3.3. Analysis of amino acid composition and fatty acids profile

The samples of PL and PLS were hydrolyzed with 6 M HCl at 110 °C for 23 h. An Aquity UPLC system (Waters, Milford, MA, USA) equipped with a photodetector was used to analyze amino acid composition as described previously by Tomczak et al. (2018). The fatty acid (FA) profile was analyzed and identified as described previously (Szczechowiak et al., 2016).

2.3.4. Rumen fermentation, methane production, and microbial population

The total gas production and methane concentrations were determined after 24 h of incubation (Cieslak et al., 2016). The volatile fatty acid (VFA) concentrations were determined by gas chromatography (GC Varian CP 3380, Sugarland, TX, USA) following a method described by Bryszak et al. (2019). The pH of the rumen samples was measured immediately after sample collection using a pH meter (Type CP-104, Elmetron, Zabrze, Poland). Ammonia concentration was determined using the colorimetric Nessler method (Cieślak et al., 2016). The loss in the weight of the incubated substrate DM after correction for the DM residue in the blank was taken as in vitro dry matter digestibility (IVDMD).

Protozoa in the fermented fluid were counted under a light microscope (Primo Star 5, Zeiss, Jena, Germany) using an appropriate volume (10 μl for *Entodiniomorpha* and 100 μl for *Holotricha*). Methanogen and total bacterial abundances were quantified by fluorescence in situ hybridization (FISH) following the procedure described previously (Szczechowiak et al., 2016). For bacteria quantification, total DNA was extracted from fermented fluid using QIAamp DNA Stool mini kit (Qiagen GmbH, Hilden, Germany) according to Szczechowiak et al. (2016). Sequences of primers specific to seven selected species and one genus of rumen bacteria are presented in a Supplementary data (STable 1). Bacteria quantification was performed with a QuantStudio 12 Flex PCR system (Life Technologies, Grand Island, NY, USA) as described previously (Szczechowiak et al., 2016).

2.4. Calculations and statistical analysis

The in-situ degradation kinetics of DM, OM, and CP were determined using the following nonlinear model according to Ørskov and McDonald (1979):

$$Dt = a + b \times (1 - e^{-ct})$$

Where D*t* is the degradation of nutrient at time t, a is the soluble fraction (fraction washed out at t = 0, obtained from bags incubated

for 0 h and corrected for particle loss); b is insoluble degradable fraction; c is the fractional degradation rate (per hour); and t is the time (h).

The effective degradability (ED) of DM, OM, and CP was determined using the following equation:

$$ED = a + (b \times c/(c + k))$$

Where k is the ruminal fractional flow rate of 0.06/h.

Chemical composition of PL (n = 8) and PLS (n = 24) were tested with an independent *t*-test, where the means of both groups were compared using the PROC TTEST procedure. In the batch culture experiment, the data were analyzed with one-way ANOVA taking treatment (i.e., feed type AS, PL, and PLS) as a main factor (n = 12 per feed type). Differences between treatments were tested using Tukey's post-hoc test. All the data from the *in sacco* degradation rate, such as DM, OM, and CP, were subjected to statistical analysis using the one-way ANOVA taking treatment (i.e., feed type AS, PL, and PLS) as a main factor (n = 24 per feed type). The least square means and Tukey's post-hoc tests were used to compare the differences between means. Data were taken as statistically different when <0.05. All values are shown as group means with pooled standard errors of means.

3. Results

3.1. Major nutrient, amino acid, and fatty acid composition of fresh (PL) and ensiled (PLS) Paulownia leaves

The ensiling process affected some of the analyzed parameters of *Paulownia* leaves. A significant decrease ($P < 0.01$) was noticed for starch content and gross energy value (Table 1). The content of amino acids increased by 22.5 % ($P < 0.003$) for essential AAs and by 23 % ($P < 0.005$) for non-essential AAs. The levels of two essential AAs, threonine and isoleucine increased significantly ($P < 0.05$; Table 2). Leucine was the most abundant AA in PLS (6.51 g AA/16 g N) whereas phenylalanine dominated in PL (6.82 g AA/16 g N). Considering nonessential AAs, the content of two of them (glycine, asparagine) increased significantly. Glutamine was the most abundant AA in PLS (6.13 g AA/16 g N) whereas serine dominated in PL (5.47 g AA/16 g N).

With regard to fatty acid content and composition, fresh *Paulownia* leaves were enriched in four FAs (C16:0, C18:2 c9c12, C18:3 c9c12c15) and contained more C14:0 ($P = 0.017$) than PLS (Table 3). The proportion of unsaturated FA (UFA) was approximately twice as high as the saturated FA (SFA) in both groups. In response to ensiling the total FA content increased by an average of 10 % whereas the abundance of total SFAs, UFAs, monounsaturated FA (MUFAs), poly unsaturated FA (PUFAs), n-3 and n-6 FAs was not altered.

3.2. Phenolic acids, flavonoids, and saponins content of PL and PLS

The ensiling process increased the concentration of both phenolic acids and flavonoids. A particular content of the main bioactive compounds identified in PL and PLS can be found in Supplementary data, Table 2. PLS contained 47.0 mg/g DM of phenolic acids and 13.0 mg/g DM of flavonoids; before the ensiling process, there was 24.6 mg/g DM of phenolic acids and 3.75 mg/g DM of flavonoids (Table 4). The flavonoids with the highest level in PL were kaempferol-O-Dhex-Hex (0.48 mg/g), kaempferol-O-Dhex (0.57 mg/g) and

Table 2
Amino acid (AA) composition (g amino acid/16 g N) of *Paulownia* leaves (PL; n = 8) and their silage (PLS; n = 24).

	PL				PLS				
	mean	SD	Min	Max	Mean	SD	Min	Max	P-value
Essential AAs	32.0	2.54	29.0	35.1	39.3	1.56	37.4	40.8	0.003
Thr	2.86	2.67	0.34	5.55	6.25	0.24	6.04	6.57	0.045
Cys	1.70	0.62	1.29	2.62	1.62	0.06	1.54	1.68	0.82
Val	2.66	0.92	1.31	3.29	3.80	0.33	3.57	4.28	0.059
Met	1.97	0.45	1.65	2.67	2.01	0.09	1.94	2.15	0.85
Ile	3.09	0.79	1.93	3.67	4.10	0.13	3.97	4.28	0.046
Leu	4.58	1.87	1.79	5.75	6.51	0.32	6.15	6.81	0.089
Tyr	4.23	0.47	3.80	4.90	4.61	0.16	4.40	4.75	0.18
Phe	6.82	2.87	5.11	11.11	6.20	0.20	5.97	6.45	0.68
His	2.47	0.19	2.29	2.74	2.67	0.23	2.40	2.97	0.24
Lys	1.63	0.63	1.26	2.57	1.47	0.08	1.38	1.56	0.63
Nonessential AAs	20.4	1.38	18.7	22.0	25.0	1.63	23.1	26.7	0.005
Arg	2.93	0.53	2.55	3.71	3.28	0.56	2.76	4.05	0.40
Asp	2.93	0.84	1.77	3.60	4.21	0.42	3.81	4.77	0.045
Pro	2.18	0.66	1.20	2.64	2.94	0.13	2.77	3.06	0.067
Glu	4.48	1.56	2.17	5.52	6.13	0.22	5.89	6.43	0.081
Ser	5.47	2.35	4.11	8.99	5.14	0.27	4.81	5.38	0.78
Gly	0.75	0.22	0.43	0.93	1.03	0.08	0.97	1.14	0.054
Ala	1.51	0.74	0.40	1.97	2.24	0.11	2.12	2.35	0.10
Total AA	52.4	3.24	49.7	54.1	64.2	3.14	60.6	67.0	0.002

Min: minimum; Max: maximum; SD: standard deviation.

Table 3
Fatty acid (FA) content (mg/g DM) of *Paulownia* leaves (PL; n = 8) and their silage (PLS; n = 24).

	PL				PLS				
	Mean	SD	Min	Max	Mean	SD	Min	Max	P-value
Total FA	16.2	3.52	10.8	19.7	17.3	3.67	10.9	22.7	0.47
C12:0	0.04	0.01	0.03	0.05	0.04	0.01	0.03	0.06	0.31
C14:0	0.08	0.03	0.04	0.13	0.11	0.03	0.07	0.15	0.017
C15:0	0.02	0.01	0.01	0.03	0.02	0.01	0.01	0.04	0.72
C16:0	3.84	0.49	3.01	4.24	4.21	0.71	2.65	5.12	0.13
C16:1	0.52	0.12	0.31	0.65	0.6	0.16	0.29	0.84	0.12
C18:0	0.57	0.09	0.44	0.69	0.64	0.14	0.45	0.85	0.11
C18:1 c9	0.73	0.36	0.39	1.34	0.81	0.4	0.56	1.80	0.62
C18:2 c9c12	2.19	0.59	1.7	3.19	2.5	0.44	1.86	3.33	0.20
C18:3c9c12c15	6.61	2.5	3.25	9.74	6.58	2.43	3.63	10.2	0.97
$\sum$ other FA[1]	1.63	0.18	1.35	1.86	1.78	0.28	1.17	2.20	0.12
SFAs[2]	5.10	0.56	4.12	5.49	5.62	0.91	3.67	6.76	0.082
UFAs[3]	11.1	3.04	6.71	14.4	11.7	2.92	7.24	16.0	0.66
MUFAs[4]	1.70	0.44	1.1	2.38	1.92	0.55	1.34	3.14	0.27
PUFAs[5]	9.43	2.91	5.59	12.7	9.76	2.73	5.9	13.7	0.78
n-6	4.72	1.13	3.76	6.63	5.36	0.93	3.98	7.01	0.17
n-3	6.80	2.53	3.42	10.1	6.78	2.46	3.71	10.34	0.98
n6/n3	0.76	0.24	0.46	1.14	0.87	0.28	0.55	1.33	0.30
n6 PUFA	2.47	0.54	1.98	3.38	2.82	0.48	2.11	3.64	0.12
n3 PUFA	6.80	2.53	3.42	10.1	6.78	2.46	3.71	10.4	0.47

SD: standard deviation; Min: minimum; Max: maximum.
[1] $\sum$ other FA: C17:0, C17:1, C20:1 t, C20:4n6, C23:0, C22:2, C24:0, C20:5n3, C24:1, C22:6n3.
[2] SFAs: saturated fatty acids.
[3] UFAs: unsaturated fatty acids.
[4] MUFAs: monounsaturated fatty acids.
[5] PUFAs: polyunsaturated fatty acids.

Table 4
Total phenolic acid, flavonoid, and saponin compositions (mg/g dry matter) in *Paulownia* leaves (PL) and its silage (PLS).

	PL (n = 4)	PLS (n = 4)
Phenolic acids	24.6 ± 0.27	47.0 ± 0.66
Flavonoids	3.75 ± 0.10	13.0 ± 0.31
Saponins	0.29 ± 0.01	$3.14 \times 10^{-4} \pm 0.01 \times 10^{-4}$

chrysoeriol 7-rutinoside (0.48 mg/g); the most abundant phenolics acids were acteoside and campneoside I (12.4 and 9.52 mg/g); following these, only the phenolic acids D-chicoric acid and 2-acetamido-4-methylphenyl acetate exceeded 1.33 mg/g and 0.81 mg/g, respectively (Supplementary data, STable 2). Phenylpropanoid glycosides were the most dominant phenolic acids in PLS; acteoside, isoacteoside, campneoside I, and epimeredinoside A exceeded 25 mg/g DM; two aglycones, kaempferol and trihydroxyflavone were the most dominant phenolic acids (4.51 mg/g DM and 3.13 mg/g DM, respectively). The level of saponins in PL and PLS was low (0.29 mg/g and 3.14×10^{-4} mg/g DM, respectively).

3.3. Batch culture experiment

Paulownia affected the rumen fermentation characteristics in a manner different to alfalfa silage. Decreases in pH and methane concentration ($P < 0.01$) were observed in PL and PLS compared to alfalfa silage (Table 5). In both PL or PLS, methane production (per gram of DM or IVDMD) decreased ($P < 0.01$), however, the greatest decrease was observed in the PLS. The ammonia concentration in PLS decreased compared to PL. The total gas production and total VFA concentrations increased in both experimental groups, but the greatest increases were noted in PL ($P < 0.01$). Concentrations of acetate, propionate, and butyrate acids significantly increased ($P < 0.01$), whereas the concentrations of iso-valerate, valerate, and the acetate-to-propionate ratio significantly decreased ($P < 0.01$) in the PL and PLS groups compared to AS. The acetate-to-propionate ratio was lowest in PL ($P < 0.04$). A significant ($P < 0.001$) decrease in *Archaea* was observed in PL and PLS. The lowest *Archaea* population was noted in PLS ($P < 0.01$). The total bacteria and *Holotricha* populations were higher in PL and PLS than in AS. The abundances of *Fibrobacter succinogenes, Butyrivibrio fibrisolvens, Prevotella* spp. were higher ($P \leq 0.03$) in the PL and PLS than the AS group. Numbers of *Megasphaera elsdenii* tended to increase ($P = 0.08$) in PL and PLS. The populations of *Ruminococcus albus, Ruminococcus flavefaciens, Streptococcus bovis,* and *Butyrivibrio proteoclasticus* were similar ($P > 0.10$) in PL and PLS in comparison with the AS.

Table 5
Effects of *Paulownia* leaves (PL) and their silage (PLS) on rumen fermentation and microbial populations *in vitro* (n = 12).

Item	AS	PL	PLS	SEM	P-value
pH	6.69^{a}	6.54^{b}	6.49^{b}	0.01	<0.01
IVDMD[1]	0.62^{b}	0.66^{a}	0.65^{a}	0.004	<0.01
Ammonia, mM	10.5^{ab}	11.0^{a}	9.77^{b}	0.18	<0.01
Total gas, ml/g DM	283^{c}	302^{a}	296^{b}	1.36	<0.01
CH_4, mmole	0.92^{a}	0.86^{b}	0.84^{b}	0.01	<0.01
CH_4, mmole/g DM	2.31^{a}	2.14^{b}	2.10^{b}	0.02	<0.01
CH_4/total gas, mmole/L	8.17^{a}	7.09^{b}	7.06^{b}	0.09	<0.01
CH_4/IVDMD, mmole/g	3.73^{a}	3.29^{b}	3.17^{c}	0.05	<0.01
Total VFA, mM	35.7^{c}	42.8^{a}	38.4^{b}	0.62	<0.01
Acetate (A)	24.5^{b}	28.3^{a}	25.3^{b}	0.36	<0.01
Propionate (P)	6.95^{c}	9.74^{a}	8.22^{b}	0.18	<0.01
Iso-butyrate	0.034	0.002	0.004	0.006	0.51
Butyrate	2.83^{c}	3.46^{b}	3.73^{a}	0.06	<0.01
Iso-valerate	0.69^{a}	0.64^{a}	0.54^{b}	0.03	<0.01
Valerate	0.63^{a}	0.59^{b}	0.57^{b}	0.01	<0.01
A/P ratio	3.53^{a}	2.91^{b}	3.09^{b}	0.04	0.04
Microbial populations					
Entodiniomorpha, 10^4/mL	2.34	2.05	2.63	0.17	0.11
Holotricha, 10^4/mL	0.18^{c}	0.51^{a}	0.24^{b}	0.02	<0.01
Total protozoa, 10^4/mL	2.53	2.56	2.87	0.07	0.09
Archaea, 10^7/mL	1.67^{a}	0.96^{b}	0.68^{c}	0.01	<0.001
Total bacteria, 10^8/mL	7.52^{b}	8.02^{a}	8.21^{a}	0.16	<0.01
Ruminococcus flavefaciens[*]	1.00	4.53	5.38	1.50	0.63
Fibrobactersuccinogenes[*]	1.00^{c}	2.59^{b}	4.34^{a}	0.44	0.03
Streptococcus bovis[*]	1.00	1.05	1.45	0.16	0.56
Butyrivibrio proteoclasticus[*]	1.00	2.51	2.07	0.69	0.63
Ruminococcus albus[*]	1.00	1.12	1.22	0.19	0.77
Butyrivibrio fibrisolvens[*]	1.00^{c}	2.79^{b}	4.82^{a}	0.34	<0.01
Megasphaera elsdenii[*]	1.00	13.8	17.3	1.75	0.08
Prevotella spp.[*]	1.00^{c}	3.52^{b}	6.93^{a}	0.59	<0.01

Within each row, means with lower case superscripts (a–c) are significantly different at $P < 0.01$; SEM: standard error of the mean.
AS: alfalfa silage; PL: *Paulownia* leaves; PLS: *Paulownia* leaves silage; IVDMD: *in vitro* dry matter digestibility; DM: dry matter; CH_4: methane; VFA: volatile fatty acid.
[*] Relative transcript abundance (ΔΔ CT).

Table 6
In sacco ruminal degradation kinetics, potential (PD) and effective (ED) degradation of *Paulownia* leaves (PL) and their silage (PLS) (n = 24).

Parameter	AS	PL	PLS	SEM	P-value
Dry matter degradation					
a	0.51^{c}	0.59^{b}	0.69^{a}	0.01	<0.001
b	0.34^{a}	0.30^{b}	0.21^{c}	0.01	<0.001
c	0.10^{b}	0.30^{a}	0.14^{b}	0.02	<0.001
PD	0.85^{c}	0.89^{b}	0.90^{a}	0.004	<0.001
ED	0.72^{b}	0.84^{a}	0.84^{a}	0.01	<0.001
Organic matter degradation					
a	0.50^{c}	0.55^{b}	0.66^{a}	0.01	<0.001
b	0.33^{a}	0.33^{a}	0.24^{b}	0.01	<0.001
c	0.10^{b}	0.30^{a}	0.15^{b}	0.02	<0.001
PD	0.83^{c}	0.88^{b}	0.90^{a}	0.01	<0.001
ED	0.71^{c}	0.82^{b}	0.81^{a}	0.01	<0.001
Crude protein degradation					
a	0.38^{c}	0.51^{b}	0.65^{a}	0.02	<0.001
b	0.48^{a}	0.20^{b}	0.15^{c}	0.03	<0.001
c	0.11^{c}	0.34^{a}	0.24^{b}	0.02	<0.001
PD	0.86^{a}	0.72^{c}	0.80^{b}	0.01	<0.001
ED	0.69^{c}	0.68^{b}	0.77^{a}	0.01	<0.001

Within each row, means with lower case superscripts (a–c) are significantly different at $P < 0.01$; SEM: standard error of the mean.
AS: alfalfa silage: PL: *Paulownia* leaves: PLS: *Paulownia* leaves silage.
a: soluble fraction; b: slowly degradable fraction; c: fractional hourly disappearance rate constant at which b is degraded; PD: potential degradability; ED: effective degradability.

3.4. In sacco experiment

Parameters of dry matter degradation differed significantly between the AS and the both *Paulownia* groups (Table 6). The PLS contained the highest soluble fraction 'a' and the lowest slowly degradable fraction 'b' ($P < 0.001$) whereas the fractional disappearance rate 'c' was higher in PL than in the PLS and AS groups ($P < 0.001$). The potential degradability of DM and OM was about 0.05–0.07 higher for PL and PLS than for AS ($P < 0.001$). With regard to OM degradation, the 'a' and PD parameters were higher in PLS ($P < 0.001$) than in the PL and AS groups. Besides, PLS displayed lowest 'b' fraction (0.24) compared to PL and control whereas the fractional disappearance rate 'c' did not differ from the DM disappearance ($P < 0.001$). For CP degradation, the PLS was characterized by the highest soluble fraction 'a' and lowest fraction 'b' ($P < 0.001$) whereas the fractional disappearance rate 'c' was significantly higher in PL ($P < 0.001$). The PD was higher in PLS and control than in PL group ($P < 0.001$). Moreover, the ED was highest in the PLS group and lowest in PL ($P < 0.001$).

4. Discussion

Leaves of many trees are a richer source of basic nutrients and bioactive substances than grasses, and are a potentially recognized feed source for ruminants year-round (Su and Chen, 2020). *Paulownia* leaves are characterized by high nutritional and medical values (Al-Sagheer et al., 2019; Adach et al., 2020). Although the main purpose of *Paulownia* cultivation is wood production (Al-Sagheer et al., 2019), a large amount of leaves estimated for about 54 tons per hectare arises as a by-product, even during pruning of trees. This plant material of high nutritive values can be successfully used for animal nutrition. The bioactive substances of plant origins may possess antinutritional characteristics, but they can also act as modulators of rumen fermentation (Bryszak et al., 2019).

To our knowledge, this is the first complete report on chemical composition and on the effect of fresh and ensiled *Paulownia* leaves on ruminal fermentation, in situ nutrient degradation, and methane production in dairy cows.

Our study has clearly shown that PL and PLS are a good source of nutrients. The CP content in PL/PLS (average 172 g/kg DM) can be considered moderate when compared with leaves of selected trees where CP content varies in a wide range from 120 g/kg in *Populus* sp. to 300 g/kg in *Moringa oleifera* (Su and Chen, 2020). It should be emphasized that the chemical composition of leaves depends on the harvest time. Leaves collected in May (as it was collected in the current study) have higher protein concentration than leaves collected in the late autumn (Ganchev et al., 2019). Because of it, NDF concentration in our plant materials was lower than that reported by Al-Sagheer et al. (2019) and (Ganchev et al., 2019). Ensiling resulted in DM loss of approximately 16 %, which is common for silage fermentation (Borreani et al., 2018). The ammonia content of PLS was lower than the average for a good quality silage suggesting a low AA deamination during ensiling. The production of ammonia can be reduced in the presence of biologically active phenolic substances (Ke et al., 2015).

To sum up, the chemical composition of both PL and PLS indicated that *Paulownia* leaves could be used as a dietary component for ruminants. The nutritional values of PLS were similar to those of alfalfa silage in terms of the average total CP content (170–190 g CP/kg DM; Ke et al., 2015). Besides, *Paulownia* is a rich source of amino acids, and the amino acid profile of the leaves is similar to that of alfalfa hay (Al-Sagheer et al., 2019). Despite proteolysis, our results showed an increase in the relative concentrations of amino acids in ensiled *Paulownia*, which could be attributed to the elevated content of phenolic acids and flavonoids, which in turn may partially inactivate the proteolytic enzymes in the ensiled materials by forming complexes with proteins (Lee et al., 2008). Non-protein N compounds in PLS may be volatized with the formation of ammonia, which may increase the relative concentrations (per 16 g N) of amino acids.

With regard to fatty acid composition, the main fatty acid in PL and PLS was C18:3 c9c12c15, while C16:0, C18:1 c9, and C18:2 c9c12 acids were the most abundant in PL and PLS. These FAs, especially polyunsaturated n-3 acids, are responsible for elevated content of health beneficial FAs in the milk, which suggests that *Paulownia* leaves can be a valuable FA source for the ruminants (Alagawany et al., 2020). Furthermore, higher level of C18:2 c9c12 in PLS may cause changes in rumen biohydrogenation. Higher levels of some FAs and SFAs in PLS comparing with PL could be explained by the loss of other parts of DM compounds such as VFA, ammonia, and CO_2 during the ensiling process (Koivunen et al., 2015). Changes in FA profile in PLS may be also related to microbial activity during ensiling process. As reported by Patra (2012), *Paulownia* leaves are rich in bioactive substances such as triterpenes, phenylpropanoid glycosides and flavonoids, especially C-geranylated flavonoids that may exert antimicrobial effects. New compounds have been isolated from some varieties of *Paulownia* (Adach et al., 2020), though only from fresh materials. In our study, ensiling increased the concentrations of several bioactive metabolites compared with the fresh leaves. PL and PLS are rich in phenolic acids and flavonoids, and PLS contains significantly more phenolic acids. This may be attributed to the loss of volatile biomass during the ensiling process (Wen et al., 2017). We showed that *Paulownia* leaves lost around 16 % of their DM during ensiling. Another reason for the substantial increase in secondary metabolites in PLS may be due to easily accessible metabolites for reagents during chemical analyses in the ensiled leaves (Szumacher-Strabel et al., 2019). Since some secondary compounds may form quite stable bindings, the ensiling process can make them more easily extractable by organic solvents. Additionally, processes that occur during ensiling include conversion of some bioactive components to other compounds, which are either absent in fresh materials or occur in trace amounts. It has been shown that the lactic acid bacteria increase enzymatic activity during the ensilage process, cause a release of bound polyphenols or other bioactive complex from plant materials, and therefore promote production and accumulation of bioactive metabolites in ensiled materials (Stefanello et al., 2018; Fan et al., 2020).

With regard to microbial populations, flavonoids have various biological properties and may stimulate bacterial growth or alter ruminal microorganisms, and thus the digestion of feed in the rumen (Zhan et al., 2017). For example, mulberry leaf flavonoids decrease ruminal populations of methanogens (Ma et al., 2017). In the present study, increases in total bacteria as well as in particular

species/genera of bacteria (*F. succinogenes, B. fibrisolvens,* and *Prevotella* spp.) were noted. However, PL and PLS decreased methane production, which may result from decreased number of *Archaea* in response to secondary metabolite activities. Inhibition of rumen methanogenesis is usually accompanied by an increase in propionate content, which was also observed in the present study. According to Mitsumori et al. (2012), high levels of H_2in the rumen shift fermentation from acetate to propionate by *Prevotella* spp. activity and we noticed an increase in the population of these bacteria. Besides, elevated propionate along with reduced acetate level can negatively affect *F. succinogenes* and *B. fibrisolvens* activity (Zhang et al., 2020). This was however not observed in the present experiment, and thus we can assume that stable acetate content can be linked to higher abundances of *F. succinogen*es and *B. fibrisolvens*.

In addition to flavonoids, another category of secondary metabolites that dominated in PLS analyzed in this study was phenylpropanoid glycosides (PPGs). PPGs are widely distributed in plants and demonstrate a wide range of biological properties (Uddin et al., 2020). Besides PPGs, acteoside, isoacteoside, and campneoside were other major components of PLS, which were also reported in other study on *Paulownia* hybrids (Adach et al., 2020). Total phenol content in PLS was as high as 60 mg/g DM, which was comparable to values reported for leaves of *Paulownia fortunei* species, and higher than in *Paulownia elongata* (He et al., 2016). All these properties increase the value of *Paulownia* leaves, both fresh and ensiled, as dietary compounds for animals. The acetoside, catalpol, and aucubin also present in *Plantago lanceolata* L. could reduce methanogen activities by decreasing the H_2 sink in the rumen (Redoy et al., 2020).

Our observations were in line with the results of Petrič et al. (2020), showing that dietary substrates containing flavonoids and phenolic acids have the potential to reduce methane emission and to cause promising changes in the rumen environment. Phenolic acids (derivatives of benzoic or cinnamic acids) are common in the essences and resins of higher plants (Bodas et al., 2012). It is known that certain phenolic acids have a toxic effect on ruminal bacteria and protozoa (Berchez et al., 2019), however, no such effects were noted in this study.

On the other hand, some studies reported inhibitory effects of phenolic acids and polyphenols on methanogenesis accompanied by improvement in certain fermentation parameters (Bodas et al., 2012). Thus, phenolic acids and flavonoids from multipurpose tropical plants may directly inhibit population of methanogens, their activity, or both (Bodas et al., 2012). Berchez et al. (2019) observed an increase in some VFAs, such as acetate. This could be explained by the adaptation of ruminal microorganisms to the phenolic acids and flavonoids, perhaps as a result of hydrogenation of phenolic acids of high toxicity to a less toxic form (Berchez et al., 2019). Our results indicated that PL and PLS exert a positive effect on rumen parameters, including reduction in methane production, which makes PL and PLS not only sources of nutrients essential to the rumen, but also a feed with a potential to reduce the negative impact of animal production on the environment.

Ensiling of PL increased the degradation of soluble OM and CP fractions and decreased the degradation of the slowly degradable OM and CP fractions. Higher degradation rates are most often associated with higher levels of NFC, which contain less fiber and are easy to ferment (Zhao et al., 2016). The NFC content in PL and PLS was 345 g/kg DM and 333 g/kg DM, respectively. A similar starch levels were found in AS and PLS (15 to 12 g/kg DM, respectively). Hence, according to Zheng et al. (2016), it is rather the presence of, for example, polysaccharide polyols, which determines higher degradation. Polysaccharide polyols are also present in leaves of other plants (Zheng et al., 2016). Sugar alcohols may increase microbial activity, degrading the substrate to higher propionic acid content (Biebl et al., 2000); this was also observed in the present in vitro study. Fernández et al. (2019) also showed greater digestibility of the OM and CP of orange leaves than that of dried alfalfa, which indicates that leaves can be a good alternative to roughage. The greater 'b' fraction in DM degradability in AS could be explained by the high CP content (215 g CP/kg DM). It should be kept in mind that saponins, phenolic acids, and flavonoids affect the degradability values of AS, PL, and PLS. Generally, the literature point of view on this aspect is not clear, as it was assumed that the energy value of the carbohydrate digested in the intestine is higher than that of the carbohydrate digested in the rumen. On the other hand, some data have shown that rumen-digested carbohydrates provide more energy to ruminants (Zhao et al., 2016). As rumen carbohydrate digestion is the main source of energy for ruminants and for microbial protein synthesis, PLS could serve as a promising dietary component for ruminants. Additionally, CP degradability increased during the ensilage process, which may suggest the effect of this process on the availability of protein (including AA) to rumen microorganisms. When using PL or PLS, protein and energy balance in the rumen should thus be considered for a greater amount of easily fermentable nonstructural carbohydrates, for example, from concentrated feeds.

5. Conclusion

Paulownia leaves are rich in CP with high proportion of essential AA and contain large amounts of phenolic substances. The use of PL and PLS mitigates methane production by reducing methanogen counts and improves rumen fermentation characteristics. The promising AA and FA profiles along with the highly effective degradability and fractional degradation rates of PL and PLS suggest that their inclusion in ruminant diets would increase nutrient and energy supply into the rumen and improve the fatty acid profile of milk. These in vitro results should be validated under in vivo conditions, as the bioactive compounds present in *Paulownia* leaves and its silage could interact with rumen microbiota in a more multidirectional manner.

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CRediT authorship contribution statement

Haihao Huang: Conceptualization, Methodology, Writing - original draft. **Malgorzata Szumacher-Strabel:** Investigation, Data

curation, Writing - review & editing. **Amlan Kumar Patra:** Writing - review & editing. **Sylwester Ślusarczyk:** Methodology, Data curation. **Dorota Lechniak:** Investigation, Writing - review & editing. **Mina Vazirigohar:** Data curation. **Zora Varadyova:** Data curation. **Martyna Kozłowska:** Visualization, Validation. **Adam Cieślak:** Supervision, Data curation, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anifeedsci.2021.115038.

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RESEARCH

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The effect of ensiled paulownia leaves in a high-forage diet on ruminal fermentation, methane production, fatty acid composition, and milk production performance of dairy cows

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Abstract

Background: The use of industrial by-products rich in bioactive compounds as animal feeds can reduce greenhouse gas production. Paulownia leaves silage (PLS) was supplemented to dairy cows' diet and evaluated in vitro (Exp. 1; Rusitec) and in vivo (Exp. 2, cannulated lactating dairy cows and Exp. 3, non-cannulated lactating dairy cows). The study investigated the PLS effect on ruminal fermentation, microbial populations, methane production and concentration, dry matter intake (DMI), and fatty acid (FA) proportions in ruminal fluid and milk.

Results: Several variables of the ruminal fluid were changed in response to the inclusion of PLS. In Exp. 1, the pH increased linearly and quadratically, whereas ammonia and total volatile fatty acid (VFA) concentrations increased linearly and cubically. A linear, quadratic, and cubical decrease in methane concentration was observed with increasing dose of the PLS. Exp. 2 revealed an increase in ruminal pH and ammonia concentrations, but no changes in total VFA concentration. Inclusion of PLS increased ruminal propionate (at 3 h and 6 h after feeding), isovalerate, and valerate concentrations. Addition of PLS also affected several populations of the analyzed microorganisms. The abundances of protozoa and bacteria were increased, whereas the abundance of archaea were decreased by PLS. Methane production decreased by 11% and 14% in PLS-fed cows compared to the control in Exp. 2 and 3, respectively. Exp. 3 revealed a reduction in the milk protein and lactose yield in the PLS-fed cows, but no effect on DMI and energy corrected milk yield. Also, the PLS diet affected the ruminal biohydrogenation process with an increased proportions of C18:3 *cis*-9 *cis*-12 *cis*-15, conjugated linoleic acid, C18:1 *trans*-11 FA, polyunsaturated fatty acids (PUFA), and reduced n6/n3 ratio and saturated fatty acids (SFA) proportion in milk. The relative transcript abundances of the 5 of 6 analyzed genes regulating FA metabolism increased.

Conclusions: The dietary PLS replacing the alfalfa silage at 60 g/kg diet can reduce the methane emission and improve milk quality with greater proportions of PUFA, including conjugated linoleic acid, and C18:1 *trans*-11 along with reduction of SFA.

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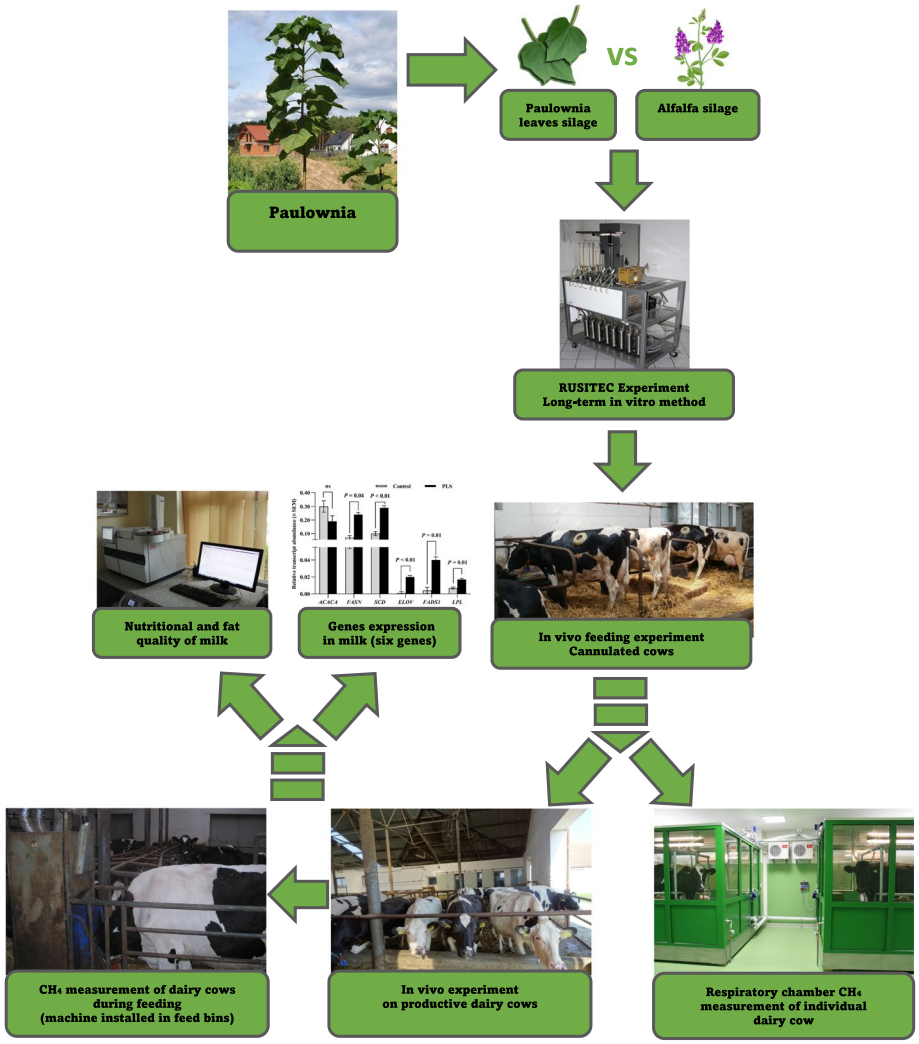
Highlights

- Paulownia leaves silage (PLS) fed to dairy cows decreased CH_4 emission.
- Dietary PLS inhibited the methanogen community and increased selected bacteria populations.
- Diet containing PLS elevated propionate proportion and decreased acetate to propionate ratio in the rumen fluid.
- Transcript abundance of genes regulating fatty acid metabolism in the milk somatic cells were increased by PLS.
- Dietary PLS elevated proportion of unsaturated fatty acids, conjugated linoleic acid, and reduced n-6/n-3 ratio in the milk.

Keywords: Dairy cow, Fatty acid composition in milk, Methane emission, Paulownia leaves

Graphical Abstract

Graphical abstract of the experimental roadmap



Introduction

Forage is one of the prerequisites to meet nutritional requirements of ruminants. Besides the forage like alfalfa silage, some by-products like pomegranate silage can be effectively utilized to protect the environment [1]. Trees of the *Paulownia* genus are known for their rapid growth [2] and thus an abundant source of wood [3]. A vast biomass of leaves becomes available as a by-product of wood processing. Leaves are rich in protein (average 175 g/kg DM), and hence can be used as an alternative source of forage for ruminants [4]. The nutritional value of Paulownia has been investigated in several studies [5–7]. Paulownia leaves are rich in bioactive compounds (BAC) such as phenolic acids and flavonoids as well as in fatty acids (FA) [4]. Besides, ensilaged paulownia leaves contain a higher concentration of BAC compared to its fresh material [4]. Our previous in vitro experiment showed that inclusion of either fresh or ensiled paulownia leaves lowered methane production by reducing the number of methanogens while improving the basic ruminal fermentation characteristics [4, 8]. Considering a high phenolic content of paulownia leaves, they can be a valuable component of dairy cow diet with an additional profit attributed to the improvement of the milk quality by altering its FA profile [4]. We hypothesized that paulownia leaves may beneficially alter ruminal fermentation variables such as pH or methane production, ammonia and volatile fatty acid concentration due to presence of phenolic compounds in PLS. Besides, the inclusion of PLS into the diet of dairy cows may affect milk composition by altering its FA profile by modifying ruminal biohydrogenation. The improvement of milk quality may result from an increase in n-3 PUFA concentration and thus a reduced n-6/n-3 ratio. However, the effect of dietary use of paulownia leaves silage (PLS) on milk production, methane mitigation, and ruminal fermentation has not been studied in dairy cows. To verify the hypothesis, we utilized the ensiled leaves of *Paulownia tomentosa* × *Paulownia fortunei* hybrid plants in an experimental design, which included in vitro and in vivo conditions. We aimed at investigating whether PLS affects (i) methane production and microbial population in vitro as well as (ii) ruminal fermentation parameters, methane production, and milk production and composition in vivo in dairy cows.

Material and methods

Diets and supplements

For the preparation of PLS, paulownia leaves (along with some twigs) were lopped from the trees (three-year-old plantations) at the end of May when the trees were shaped. Leaves were wilted for 8 h and were ensiled using the biological additive (Agricol Sil, Microferm, UK) in 60 kg plastic drums (diameter 40 cm and height 61 cm) according to Huang et al. [4]. The alfalfa was harvested at the second cut at the beginning of June. The alfalfa fresh materials after wilting approximately to 35% of dry matter were chopped to a particle length circa 2.5 cm and ensiled using the same biological additive as paulownia silage. One gram of the additive contained 10^{11} colony-forming units of *Lactobacillus plantarum* DSMZ 16,627 and *Pediococcus acidilactici* NCIMB 30,005, as well as an enzyme-producing strain, *Lactobacillus paracasei* NCIMB 3015. The closed drums were stored for 8-9 weeks in the case of both plants. The chemical composition of PLS and AS is presented in Table 1. The following fermentation parameters of PLS and AS were observed: pH, 4.75 vs. 4.50; NH_3-N, 79.4 vs. 32.3 g/kg total nitrogen; lactic acid, 21.2 vs. 34.9 g/kg DM; acetic acid, 7.04 vs. 19.1 g/kg DM; propionic acid, 1.29 vs. 1.09 g/kg DM; butyric acid, 1.19 vs. 2.43 g/kg DM, respectively.

In vitro experiment (Exp. 1)

The in vitro experiment was conducted using the Rusitec system equipped with four fermenters of 1 L volume each, following the procedures described by Szczechowiak et al. [9]. Rumen fluid and solid digesta for the in vitro experiment were collected 3 h before the morning feeding from four rumen-cannulated (Bar Diamond, Parma, Idaho, USA) multiparous Polish Holstein–Friesian dairy cows (630 ± 25 kg body weight) at their 3rd month of lactation. The following diets were tested: control diet (CON) and three PLS diets. The CON diet contained the following forages: corn silage (388 g/kg DM), alfalfa silage (82 g/kg DM), and meadow grass silage (91 g/kg DM; Table 1). The PLS diets contained paulownia silage that replaced alfalfa silage at 25%, 50%, and 75%, which corresponded to the PLS content in the diet at the level of 20, 40, and 60 g/kg DM (Table 1). Ruminal fluid donor cows received the CON diet twice a day ad libitum (Table 1). To meet the nutrient requirements of mid-lactation dairy cows (600 kg body weight, 110 d in milk, and 34 kg/d milk production, 4% of fat content in milk), the in vivo diets were prepared using the FeedExpert software (Rovecom, Hoogeveen, Netherlands). The in vitro experiment was designed in a completely randomized design comprising four diets and three replicates. From d 6 to 10 of each run, fermentation fluid samples were collected under anaerobic conditions from each vessel 3 h before feeding time. The collected fluid samples were analyzed for pH, volatile fatty acids (VFA), ammonia concentration, protozoa, bacteria and methanogen counts. For fatty acids analysis, samples were collected directly from the effluent vessels during

Table 1 Ingredients and chemical composition of experimental diets ($n=4$) used in Rusitec system and in vivo experimets[a] and chemical composition of paulownia leaves silage (PLS; $n=4$) and alfalfa silage (AS; $n=4$)

Item	PLS	AS	Treatments[b]			
			CON	PLS, g/kg DM		
				20	40	60
Ingredient composition, g/kg DM						
Corn silage	-	-	388	385	386	386
Alfalfa silage	-	-	82	68	47	26
Paulownia silage	-	-	0	21	39	60
Meadow grass silage	-	-	91	90	90	90
Beet pulp	-	-	103	103	103	103
Brewer's grain	-	-	95	94	95	95
Concentrate[c]	-	-	119	118	119	119
Rapeseed meal	-	-	108	107	107	107
Mineral and vitamin premix[d]	-	-	14	14	14	14
Forage to concentrate ratio	-	-	76:24	76:24	76:24	76:24
Chemical composition[e], g/kg DM						
DM, g/kg as fed	279	299	425	423	422	419
OM	869	874	909	907	904	900
aNDF	359	350	347	346	349	351
CP	174	218	161	160	158	158
EE	27.5	20.0	27.1	29.8	30.5	29.5
Total phenolic compounds[f]	60.0	-	0.00	1.20	2.40	3.60
VEM[g]	785	667	948	945	943	942
Fatty acid composition, g/100 g total FA						
C12:0	0.19	2.18	0.31	0.24	0.31	0.31
C14:0	0.71	1.90	0.47	0.40	0.50	0.48
C16:0	27.4	26.5	22.3	21.3	21.2	21.5
C16:1 *cis*-9	3.89	1.36	0.74	0.78	1.03	0.99
C18:0	4.16	4.01	2.63	2.55	2.16	2.82
C18:1 *cis*-9	5.26	4.76	20.3	19.7	18.9	17.8
C18:2 *cis*-9.*cis*-12	16.2	20.4	44.4	45.2	44.6	44.7
C18:3 *cis*-9.*cis*-12.*cis*-15	42.2	38.8	8.85	9.83	11.3	11.4

[a] In the in vitro experiments the diets were as a total mixed ration (TMR)

[b] *CON* control diet, *PLS* paulownia leaves silage diet, PLS was used at 20, 40 and 60 g/kg DM of diet replacing alfalfa silage

[c] Declared to contain (as g/kg of DM in concentrate) OM (910), aNDFom (240), CP (17.5), and EE (31)

[d] Declared to contain (g/kg of DM) Na (123), Ca (100), Mg (45), P (42), K (20), S (18), Co (14), Cu (5.0), Zn (2.8), Mn (1.4), Fe (1.05), F (0.42), I (0.028), Se (0.018), biotin (0.008); (IU/kg), vitamin A (200,000), vitamin D_3 (40,000), and vitamin E (1200)

[e] *DM* dry matter, *OM* organic matter, *aNDF* neutral detergent fiber analyzed with α-amylase, *CP* crude protein, *EE*: ether extract. Additionally, in the case of PLS and AS, the following fermentation parameters were performed: pH, 4.75 vs. 4.50; NH_3-N, 79.4 vs. 32.3 g/kg total nitrogen; lactic acid, 21.2 vs. 34.9 g/kg DM; acetic acid, 7.04 vs. 19.1 g/kg DM; propionic acid, 1.29 vs. 1.09 g/kg DM; butyric acid, 1.19 vs. 2.43 g/kg DM, respectively

[f] The content of total phenolic compounds have been calculated based on previous study of Huang et al. [4]

[g] VEM = feed unit net energy lactation; calculated using the FeedExpert software

bag replacements. Before feeding time (once a day), the fermentation gas was collected in a gas-tight collection bag (Tecobag 81; Tesseraux Container, Bürstadt, Germany) for the methane concentration measurement. The DM degradability was determined by analyzing feed residues in pre-feeding nylon bag samples for the last 5 d of each run (d 6 to 10).

In vivo experiment using rumen-cannulated dairy cows (Exp. 2)

In Exp. 2, four multiparous cannulated Polish Holstein–Friesian dairy cows (625 ± 20 kg body weight; 4-5th month of lactation) were assigned to two dietary treatments (CON vs. PLS60) with two cows in each treatment in a replicated 2 × 2 crossover design. Based on

the results from Exp. 1 (mainly pH, total VFA concentration, methane production, and methanogens population), the CON and PLS diets containing the higher level of PLS, i.e., 60 g/kg DM (PLS60) were implemented in Exp. 2. Cows were fed two times a day. Each period lasted for 36 d, with a 21-d adaptation (where cows were also introduced and accustomed to the conditions of the respiration chambers; temperature 12 to 22 °C and the humidity of 50% to 70%) and a 15-d sampling period (5 d of rumen fluid collection and 10 d for gases collections). The cows were housed in tie stalls with rubber mats with individual feeding and had free access to water and salt blocks during the adaptation and sampling (without the period in which the cows were kept in the respiration chambers). The ruminal fluid was collected from each cannulated cow from three locations (top, bottom, and middle) of the midventral sac of the rumen before morning feeding (0 h) and 3 h and 6 h after morning feeding [10]. Rumen samples (about 400 g/animal) were filtered through a two-layer cheesecloth and analyzed for pH value, ammonia and VFA concentrations, and FA profile. For protozoa counting, about 100 g/animal of the rumen content was mixed with an equal amount of 8% formaldehyde solution (w/w), strained through a two-layer cheesecloth into 10 mL polypropylene tube with a screw cup, and stored at 8 °C in a refrigerator until analysis. Quantification of total bacteria and methanogens was carried out only on rumen fluid sampled 3 h after morning feeding. For microbial analysis, rumen content (300 g) was strained through a two-layer cheesecloth into 100 mL polypropylene box, mixed, transferred into two cryotubes of 4.5 mL and frozen in liquid nitrogen. Samples were stored at − 80 °C until further analyses.

Feed intake, feed residue, and amount of feces were recorded daily from individual cows kept in respiratory chamber (SPA System, Wroclaw, Poland) during the sampling period (d 27 to 36) to determine the total-tract degradability coefficients. The feed and feces subsamples (about 5% wt/wt) were stored at -20 °C for DM, organic matter (OM), neutral detergent fiber (NDF), and CP analyses. The total tract nutrient degradability was calculated as [(nutrient intake − nutrient in feces)/nutrient intake] × 100.

In vivo experiment using commercial dairy cows (Exp. 3)

In Exp. 3, 16 multiparous lactating Polish Holstein–Friesian dairy cows [600 ± 30.4 kg body weight, 2.4 ± 0.45 parity, 160 ± 32 d in milk, and 33 ± 2.1 kg/d milk production; (mean ± SD)] were assigned to two dietary treatments (CON vs. PLS60) with eight cows in each treatment in a replicated 2 × 2 crossover design. Each period consisted of a 21-d adaptation period followed by a 5-d sampling period with a total of 26 d. Cows were randomly assigned to one of the two dietary groups ($n = 8$): control (CON) and experimental diet (PLS 60) and kept separately in a dedicated area of a barn. The two groups (8 CON and 8 PLS60) had separated access (gate with access control) to a computer-controlled feeder station (De Laval, type FP 204, Tumba, Sweden). The concentrate supplement, contained (as g/kg of DM in concentrate) OM (910), aNDFom (240), CP (17.5), and EE (31), was supplied in both groups at 2.75 kg/d/cow (5 times a day). The rest of the diet was offered twice a day (at 06:00 and 18:00 h) as a partial mixed ration (PMR) in individual feeding boxes located on the feeding table. Due to the limitations of running the experiment under production conditions, the cows were supervised by two workers for 18 h, who ensured that each cow consumed feed from an appropriate feeding box (CON or PLS60). For the remaining 6 h, the feeding boxes were closed. The control PMR contained (g/kg of DM): corn silage (441); alfalfa silage (93), meadow grass silage (103); beet pulp (118), brewer's grain (108); rapeseed meal (122); mineral and vitamin premix (16) whereas experimental PMR contained (g/kg of DM): corn silage (438); alfalfa silage (29), paulownia leaves silage (68), meadow grass silage (102); beet pulp (117), brewer's grain (108); rapeseed meal (122); mineral and vitamin premix (16). The nutritive value of the two diets (CON and PLS60) was the same. Cows had access to clean water ad libitum. The dry matter intake was measured daily for the last 5 d (d 22 to 26) of the experiment by weighing the individual amounts of feeds offered and leftovers in the feeding boxes. Feces were individually collected from each cow directly after defecation and the floor was kept clean. Subsamples (about 5% wt/wt) were collected and stored at -20 °C until analysis. Total tract nutrient digestibility was calculated using the method described for Exp. 2. Cows were milked twice a day at 5:30 and 17:30 h in a herringbone milking parlor with 8 milking units, spending a maximum of 1 h/d outside the pen for milking. Milk samples were collected from all cows at each milking during the sampling period (d 22 to 26) based on the proportion of morning and evening yield. The morning milk samples were stored at 4 °C until the evening samples were taken. Subsequently, milk samples were prepared in three equal parts: first part was used to analyze milk basic constituents, second part was stored at -20 °C for FA analysis, and the third part was quickly stored in liquid nitrogen for gene expression analysis.

Sample analysis

Feed and feces samples were analyzed according to AOAC methods [11] for DM (method no. 934.01), ash (method no. 942.05), CP (Kjel-Foss Automatic 16,210 analyzer, Foss Electric, Hillerød, Denmark; method no.

976.05), ether extract (EE; Soxhlet System HT analyzer; Foss Electric, Hillerød, Denmark; method no. 973.18) and neutral detergent fiber (aNDF) with amylase and sodium sulfite and expressed without residual ash (Fibertech 1020 Analyzer; Foss., Analytical AB, Höganäs, Sweden; method according Van Soest et al. [12]. Organic matter was obtained by subtracting ash from DM. Concentration of total phenolic compounds in the diets (Table 1) was calculated by utilizing data for ensiled paulownia leaves published by Huang et al. [4]. The same batch of ensiled paulownia leaves was used in the present study. The silage pH was determined using a pH meter (Elmetron, Type CP-104, Zabrze, Poland). Ammonia was estimated according to the Nessler method [13]. Lactic, acetic, propionic, and butyric acids were determined using a High Performance Liquid Chromatography (Waters 2690, Santa Clara, CA, USA) equipped with Waters 2487 Dual λ detector and Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, Warsaw, Poland). The quantitative and qualitative evaluations of individual peaks were made using the external standard method prepared by mixing individual fatty acids purchased from Supelco (Poznan, Poland) and analysed with the Millennium 2001 software (version 2.15, Waters Corporation, Manchester, England).

The pH of ruminal fluid was measured immediately after sample collection using a pH meter (Elmetron, Type CP-104, Zabrze, Poland). The ammonia concentration was determined using the colorimetric Nessler method and the VFA was analyzed using gas chromatography (GC Varian CP 3380, Sugarland, TX, USA) as described earlier [9, 13]. In the first two experiments, the content of DM degradability (g/kg) was determined by the difference between the initial feed substrate weight and residue weight after incubation.

Protozoa counts in the fermented fluid were carried out under a light microscope (Primo Star 5, Zeiss, Jena, Germany) using an appropriate volume (10 μL for Ophryoscolecidae and 100 μL for Isotrichidae). In Exp. 2, the protozoan genera and species were identified according to size and shape of cells, skeletal plates (if present), macronucleus, and arrangement of ciliature [14]. The methanogens and total bacteria were quantified by fluorescence in situ hybridization (FISH), following the procedure described previously [15] with some modification. Briefly, 6 mL of sterile paraformaldehyde-phosphate buffered saline (PBS; pH 7.2) was added to 2 mL of ruminal fluid in a stomacher bag. The mixture was homogenized in a stomacher (Interscience, Saint-Nom-la-Breteche, France) for 2 min. Afterwards, 2 mL of the homogenized mixture was transferred to the eppendorf tubes and fixed with 4% (w/v) sterile paraformaldehyde-PBS for 3 h at 48°C. Mild sonication (two times for 30 s using Hielscher Ultrasonics, Teltow, Germany) was done to avoid the formation of clusters and to optimize homogenization of the samples [16]. The prepared samples were pipetted onto 0.22 μm polycarbonate filters (Frisenette K02BP02500) and vacuumed (Vaccum KNF Vacuport-Neuberg). After vacuuming, the filters were transferred onto cellulose disks for dehydration in ethanol series (50%, 80%, and 90%, 3 min each). For each sample, a series of identical filters were prepared to allow the determination of optimal hybridization. Hybridizations were carried out in 50 μL of hybridization buffer (0.9 mol/L NaCl; 20 mmol/L Tris/HCl, pH 7.2; 0.01% SDS) containing oligonucleotide probes for all methanogens (S-D-Arch-0915-a-A-20) and two order-specific probes (S-O-Mmic-1200-a-A-21 Methanomicrobiales and S-F-Mbac-0310-a-A-22 Methanobacteriales) [17]. After hybridization, the filters were washed with a washing buffer (20 mmol/L Tris/HCl, pH 7.2; 0.01% SDS; 5 mmol/L EDTA) for 20 min at 48 °C. The filters were rinsed gently in distilled water, air-dried, and mounted on object glasses with VectaShield (Vector laboratories nr. H-1000) anti-fading agent containing DAPI (4′,6-diamidino-2-phenylindole). To distinguish the total count of bacteria (DAPI) from methanogens in the ruminal fluid, filters were maintained at 4 °C for one h in the dark until visualization using an Axio Imager M2 microscope (Carl Zeiss Iberia, Madrid, Spain). Besides bacteria counts, relative changes in the population of seven selected species (*Ruminococcus flavefaciens, Fibrobacter succinogenes, Streptococcus bovis, Butyrivibrio proteoclasticus, Ruminococcus albus, Butyrivibrio fibrisolvens, Megasphaera elsdenii)* and two genera (*Prevotella* spp., *Lactobacillus* spp.) of ruminal bacteria were determined by quantitative real-time PCR (qPCR). For this purpose, total DNA was extracted from the ruminal fluid using QIAamp DNA Stool mini kit (Qiagen GmbH, Hilden, Germany) according to Szczechowiak et al. [9]. Sequences of primers specific to bacterial genera and species are given in a Supplementary data (Table S1). The specificity of primers (Table S1) was confirmed in the GenBank Database using the BLAST program. The quantitative analysis of particular bacteria was performed with a known starting concentration of bacterial DNA (25 ng/μL) using the QuantStudio 12 Flex PCR system (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) [9]. The Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) was used for PCR amplification. The reaction mixture (final volume of 10 μL) contained 4 μL of the 2 × Mastermix, 25 ng of template DNA and 0.5 mol/L of each primer. Amplification involved one cycle at 95 °C for 10 min for initial denaturation, 45 cycles of 95 °C for 15 s followed by annealing at temperatures depending upon the individual bacteria, and then primer extension at 60 °C for

62 s. The fluorescent product was monitored in the last step of each cycle. To determine the amplicon specificity, melting analysis was performed after a single amplification (0.1 °C × s^{-1} increment from 65 °C to 95 °C with fluorescence collection at 0.1 °C intervals). Additionally, the size of amplicons was verified by gel electrophoresis. The size of total bacterial populations was referred to the calculated copy number concentrations of the 16S rRNA (*rrs*) gene [18]. The absolute abundance of bacterial DNA was expressed as a number of *rrs* gene copies/mL of ruminal sample.

In the first experiment, the methane concentration was determined using gas chromatograph (SRI PeakSimple 310; Alltech, PA, USA) fitted with Carboxen 1000 column (Supelco, Bellefonte, USA) and thermal conductivity detector according to Szumacher-Strabel et al. [19]. In the in vivo experiments, the methane and carbon dioxide concentration were measured using two separate NDIR (nondispersive infrared spectroscopy) systems (one system per gas) operating in the near-infrared spectrum (detector 1210 Gfx Servomex 4100, Servomex, Crowborough, UK). In Exp. 2, two respiration chambers were used for monitoring of methane production. Briefly, two open-circuit respiration chambers (W × L × H: 300 cm × 400 cm × 220 cm; SPA System, Ltd., Wroclaw, Poland) were used to measure CH_4 and CO_2 over 10 d (d 27 to 36). During the sampling period (from d-27 to 36 of the experiment), individual cows were transferred into a respiratory chamber by daily rotation in order to determine the direct CH_4 emission for 23.5 h consecutively. The time of milking (approximately 30 min) that accompanied the morning and evening feedings was not included in the gas emission calculations. Finally, each cow was monitored for 5 d. During the adaptation period (from d 11 to d 20) to decrease stress, cows were getting accustomed to the respiratory chambers. Cows were restrained within the chambers by a neck yoke on a dedicated platform (180 cm × 126 cm) covered with a rubber mat and had free access to fresh water and salt blocks. Emission of CH_4 and CO_2 was determined using two NDIR analyzers operating in the near-infrared spectrum (SERVOMEX 4100, SERVOMEX Ltd, UK, detector 1210 Gfx). Measurements were taken every 2-s interval. Two measuring channels were used: the concentration of CO_2 in the range of 0–2.5% (0–48,450 mg/m^3) and the CH_4 concentration in the range of 0–1000 ppm (0–706 mg/m^3). Samples were collected and ducted to the analyzer via a polyethylene tube with an 8 mm diameter. The sampling rate was 0.6 L/min. Before starting the experiment, analyzers were calibrated using calibration gasses: nitrogen N 5.0 (99,999 vol % purity) and 1210 ppm CH_4 in nitrogen. The analyzer was equipped with 0.17 L cuvette with 540 mm optical track length for CH_4 and 0.012 L cuvette with 154 mm optical track length for CO_2. In Exp. 3, methane and carbon dioxide concentrations were measured by the same type of detectors during the feeding of concentrate in the feeder station for the last 4 d of the experiment [20]. Air samples were continuously collected from the feed bins in the feeder station at 15 L/min via an 8-mm diameter polyethylene tube and connected to detectors.

The FAs concentrations in collected samples (feed, ruminal fluid, and milk) were determined by gas chromatograph (456-GC, Bruker, USA) with fused-silica capillary column (100 m × 0.25 mm; overlaid with 0.25 μm Agilent HP; Chrompack CP7420; Agilent Technologies, Santa Clara, CA, USA) and flame ionization detector [9].

Daily milk yields were recorded using a milk meter (WB Ezi-Test Meter 33 kg; True-Test, Manukau, New Zealand). The milk composition was measured by infrared analysis (MilkoScan 255 A/S N, FossElectric, Hillerød, Denmark). For gene expression in milk somatic cells, total RNA was isolated from 10 mL milk samples frozen in liquid nitrogen using previously published procedure [9]. Briefly, milk samples after thawing at 4 °C were centrifuged in 15 mL tubes for 10 min at 3000 × *g*. The supernatant was discarded, and the pellet was dissolved in 1 mL TriPure reagent (Roche) and incubated 5 min. After incubation, 200 μL of chloroform were added and shaken vigorously for 30 s. After 10 min incubation at room temperature, the sample was centrifuged for 15 min at 12,000 × *g*. The clear phase was transferred to a new tube, 0.5 mL isopropanol was added and incubated for 10 min at room temperature. The next steps involved RNA precipitation with a 75% ethanol and drying on a 40 °C thermoblock. The RNA was resuspended in DEPC treated water following spectrophotometric measurement (Nanodrop c2000, Thermo Scientific, USA) of its concentration and purity. A reverse transcription reaction (RT) was performed using a Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's protocol. Each sample contained equal concentrations of RNA. The RNA mix composed of RNA (300 ng), random hexameters, oligodT (60 μmol/L and 2.5 mmol/L respectively) and water was incubated at 65 °C for 10 min. Next, reverse transcriptase, RNAse inhibitor, dNTP and buffer were mixed and added to the RNA mix to a final volume of 20 μL. The RT conditions were as follows: 25 °C for 5 min, followed by 42 °C for 45 min and 85 °C for 5 min. Resultant cDNA was stored at − 20 °C until further analyses. The mRNA expressions of six genes encoding enzymes regulating FA metabolism [acetyl-CoA carboxylase 1 (*ACACA*), fatty acid synthase (*FASN*), lipoprotein lipase (*LPL*), stearoyl-CoA desaturase (*SCD*), fatty acid desaturase 1 (*FADS1*) and fatty acid elongase 5 (*ELOVL5*)] were measured in milk somatic

cells using previously published primer pairs [21, 22]. Each gene was analyzed in technical duplicates using a LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland) and a LightCycler 480 Sybr Green I Master reagent (Roche Diagnostics, Basel, Switzerland).

Statistical analysis

The data of the Exp. 1 (Rusitec) were analyzed using a mixed model procedure (PROC MIXED) of SAS (university edition, version 9.4; SAS Institute, Cary, NC, USA). The dietary treatment was considered as the fixed effect, experimental run as the random effect, and the day (6 to 10 d) as the repeated factor. The linear, quadratic and cubic contrasts were used to determine the effect of PLS dose. In Exp. 2 (cannulated cows), ruminal fermentation and FA data were analyzed using PROC MIXED (ver. 9.4, SAS Institute Inc., Cary, NC) for a crossover design with a model containing group (dietary treatment sequence), period, and treatment as main effects, sampling time as repeated measures, and cow as a random effect. The model for the bacteria, methanogens and degradability analyses contained cow, group, period, and treatment as a main effect. In Exp. 3 (productive dairy cows), the data were subjected to analysis of variance, considering the crossover design, testing the effect of treatment (CON and PLS60), group (dietary treatment sequence), period as fixed effects and cows within a group as a random effect. The analysis of milk production, component yields, and composition was performed on the mean values of the milk variables obtained from two sampling points per day (morning and evening milking). The analysis of FA proportion in the milk as well as expression of six genes was conducted on the mean values of pooled samples from morning and evening milking. The results were tested with an independent *t*-test where the means of both groups were compared through PROC TTEST procedure. The results were considered significant when the *P*-values were lesser than 0.05. All values are shown as the means with pooled standard errors of means.

Table 2 The effect of paulownia leaves silage (PLS) on in vitro ruminal fermentation and methane production ($n=4$) (Exp.1)

Parameters[a]	CON	PLS, g/kg DM[b]			SEM	Contrast[c]		
		20	40	60		L	Q	C
Rumen fermentation								
Redox potential, mV	-313	-314	-316	-319	1.27	0.22	0.77	0.87
pH	6.28	6.52	6.62	6.71	0.02	< 0.01	< 0.01	0.06
NH_3, mmol/L	9.09	9.30	12.5	12.8	0.25	< 0.01	0.89	< 0.01
Total VFA, mmol/L	63.5	64.9	66.5	69.5	0.48	< 0.01	0.19	0.68
VFA, mol/100 mol								
Acetate (A)	60.5	57.8	57.4	55.6	0.37	< 0.01	0.38	0.10
Propionate (P)	16.9	18.3	18.8	19.7	0.21	< 0.01	0.27	0.25
Isobutyrate	0.85	0.96	1.04	1.11	0.02	< 0.01	0.08	0.63
Butyrate	13.1	14.0	14.3	14.6	0.12	< 0.01	0.22	0.44
Isovalerate	2.49	2.44	2.41	2.40	0.04	0.29	0.69	0.96
Valerate	6.05	6.44	6.44	6.59	0.08	< 0.01	0.28	0.29
A/P ratio	3.56	3.19	3.04	2.84	0.05	< 0.01	0.18	0.31
Degradability, g/kg DM								
DM	538	540	562	564	6.07	0.18	0.99	0.52
OM	570	573	593	595	5.70	0.18	0.99	0.52
CP	569	574	578	582	2.74	0.13	0.97	0.93
NDF	437	420	443	440	6.67	0.71	0.64	0.31
Total gas and methane production								
TGP, mL/d	3722	3881	3958	4151	25.7	< 0.01	0.71	0.31
CH_4, mmol/L	9.45	8.10	6.08	5.94	0.21	< 0.01	0.03	0.04
CH_4, mmol/g DMD	1.58	1.29	0.94	0.92	0.04	0.02	0.03	0.17
CH_4, mmol/g NDFD	3.57	3.05	2.26	2.04	0.10	0.26	0.39	0.15

[a] *VFA* volatile fatty acid, *DM* dry matter, *Om,* organic matter, *CP* crude protein, *NDF* neutral detergent fiber, *TGP* total gas production, *DMD* dry matter degradability *NDFD* neutral detergent fiber degradability

[b] *CON* control diet, *PLS* paulownia leaves silage diet, *PLS* was used at 20, 40, and 60 g/kg DM of diet replacing alfalfa silage

[c] *L* linear response, *Q* quadratic response, *C* cubic response. The results are considered to be significantly different at $P \leq 0.05$

Results

Exp. 1 (Rusitec study)

Ruminal pH increased linearly and quadratically ($P < 0.01$) with increasing concentrations of PLS with the greatest pH at the highest PLS inclusion (Table 2). The inclusion of PLS in diets increased the ammonia concentration linearly and cubically ($P < 0.01$) and total VFA concentration linearly ($P < 0.01$). Molar proportion of acetate and acetate to propionate ratio decreased ($P < 0.01$) linearly; however molar proportions of other VFA (except isovalerate) increased linearly with increasing levels of PLS ($P < 0.01$). Degradability of nutrients was not affected by the PLS inclusion in diets. The total gas production increased ($P < 0.01$) linearly when PLS was included into the diet. Daily methane concentration decreased ($P < 0.05$) linearly, quadratically, and cubically with increasing doses of PLS in diets. Methane concentration per unit of degraded DM decreased linearly and quadratically ($P < 0.05$) as the PLS dose increased. The inclusion of PLS into the diet resulted in changes ($P < 0.05$) of most microbial populations in a linear pattern (Table 3). Total protozoa and Ophryoscolecidae populations decreased linearly ($P < 0.01$) whereas Isotrichidae counts increased linearly ($P < 0.05$) with increasing PLS levels. Total archaea, Methanobacteriales and Methanomicrobiales populations were decreased ($P < 0.01$) linearly by the higher replacement of alfalfa silage with PLS in the diet. The increasing PLS supplementation caused linear increase of *Ruminococcus flavefaciens, Fibrobacter succinogenes, Streptococcus bovis*, *Prevotella* spp., *Butyrivibrio fibrisolvens,* and *Megasphaera elsdenii* ($P \leq 0.05$) abundances. Besides, *M. elsdenii* showed quadratic and cubical responses ($P < 0.05$).

In vivo experiment (using cannulated cows)

Inclusion of PLS resulted in an increase ($P < 0.01$) in pH and ammonia concentration in the rumen. Besides, pH values were post-feeding time dependent ($P < 0.01$) whereas ammonia concentration showed treatment × time interaction (Table 4). The inclusion of PLS decreased the molar proportion of acetate ($P < 0.01$), but increased the molar proportion of propionate (3 h and 6 h after feeding; $P < 0.01$), isovalerate ($P < 0.05$) and valerate ($P < 0.01$). Molar proportions of most individual VFA showed time-dependent variations ($P < 0.05$). A/P ratio was lower in the PLS group compared to the control group in 3 h and 6 h after morning feeding ($P < 0.01$). Ammonia concentration was affected ($P < 0.01$) by time × treatment interaction, which increased in the PLS diet compared with the control diet ($P < 0.01$) at 3 and 6 h, but not at 0 h.

Total Isotrichidae, *Dasytricha ruminantium*, *Isotricha prostoma* and *Isotricha intestinalis* populations increased by feeding the PLS diet. The abundances were affected by treatment × time interaction ($P < 0.01$), except for *Isotricha intestinalis*. *Ostracodinium gracile* and *Polyplastron*

Table 3 The effect of paulownia leaves silage (PLS) on in vitro ruminal microbial population ($n = 4$) (Exp.1)

Parameters	CON	PLS, g/kg DM[a]			SEM	Contrast[b]		
		20	40	60		L	Q	C
Total bacteria, × 10^8/mL	1.72	1.58	1.63	1.53	0.08	0.20	0.85	0.40
Total protozoa, × 10^3/mL	14.0	12.3	11.9	10.7	0.25	< 0.01	0.62	0.27
Ophryoscolecidae, × 10^3/mL	13.2	11.5	10.8	9.17	0.27	< 0.01	0.92	0.36
Isotrichidae, × 10^3/mL	0.76	0.79	1.15	1.48	0.05	< 0.01	0.05	0.27
Total archaea, × 10^6/ mL	3.29	2.86	2.55	2.17	0.13	< 0.01	0.87	0.84
Methanobacteriales, × 10^6/mL	2.46	2.15	1.96	1.68	0.09	< 0.01	0.93	0.79
Methanomicrobiales, × 10^5/mL	2.40	2.13	2.02	1.66	0.09	< 0.01	0.79	0.55
*Ruminococcus flavefaciens**	0.73	0.65	3.03	5.03	0.80	0.02	0.41	0.75
*Fibrobacter succinogenes**	0.19	0.55	0.51	1.83	0.22	0.05	0.33	0.48
*Streptococcus bovis**	1.06	2.00	5.61	18.85	2.55	< 0.01	0.11	0.59
Prevotella spp.*	3.26	6.88	13.78	20.75	2.06	< 0.01	0.40	0.28
*Butyrivibrio proteoclasticus**	1.68	3.18	4.21	6.70	1.06	0.11	0.14	0.53
*Ruminococcus albus**	0.21	0.27	1.11	1.42	0.16	0.65	0.13	0.89
*Butyrivibrio fibrisolvens**	0.40	0.44	1.52	4.25	1.04	< 0.01	0.98	0.52
Lactobacillus spp.*	2.15	3.44	8.24	10.06	0.94	0.20	0.61	0.96
*Megasphaera elsdenii**	0.73	0.65	3.03	5.03	0.80	< 0.01	< 0.01	0.02

[a] *CON* Control diet, *PLS* paulownia leaves silage diet

[b] *L* linear response, *C* cubic response

* Abundance (log_{10} no. of copies of rrs gene/mL of buffered rumen sample)

Table 4 The effect on replacing alfalfa silage with paulownia leaves silage (60 g/kg) on ruminal fermentation characteristics measured in rumen-cannulated cows ($n=4$) (Exp. 2)

Variables[1]	0 h[2]		3 h[2]		6 h[2]		Treatment		SEM	*P*-value[4]		
	CON[3]	PLS[3]	CON	PLS	CON	PLS	CON	PLS		T	H	T × H
pH	5.93	6.07	5.92	6.13	6.09	6.18	5.98	6.13	0.02	< 0.01	< 0.01	0.22
NH_3, mmol/L	8.73^{b}	8.57^{b}	7.84^{b}	11.2^{a}	6.75^{c}	12.4^{a}	7.80	10.7	0.41	< 0.01	0.50	< 0.01
Total VFA, mmol/L	106	108	106	104	107	107	106	106	0.53	0.64	0.33	0.44
VFA, mol/100 mol												
Acetate (A)	64.5^{a}	64.4^{a}	63.0^{a}	60.2^{b}	63.1^{a}	59.2^{b}	63.5	61.2	0.25	< 0.01	< 0.01	< 0.01
Propionate (P)	22.1^{c}	21.1^{c}	22.4^{c}	24.8^{a}	23.1^{bc}	24.8^{a}	22.6	23.6	0.19	< 0.01	< 0.01	< 0.01
Isobutyrate	0.67	0.75	0.70	0.74	0.65	0.77	0.67	0.75	0.03	0.22	0.96	0.85
Butyrate	9.88^{c}	10.6^{b}	11.1^{a}	10.7^{b}	10.6^{b}	11.8^{a}	10.5	11.0	0.12	0.02	< 0.01	< 0.01
Isovalerate	1.10^{b}	1.52^{a}	1.13^{b}	1.80^{a}	1.10^{b}	1.84^{a}	1.11	1.72	0.04	< 0.01	0.03	0.04
Valerate	1.66^{b}	1.67^{b}	1.66^{b}	1.81^{a}	1.38^{c}	1.86^{a}	1.57	1.78	0.03	< 0.01	0.08	< 0.01
A/P ratio	2.93^{a}	3.06^{a}	2.83^{a}	2.47^{b}	2.75^{a}	2.40^{b}	2.84	2.64	0.03	< 0.01	< 0.01	< 0.01
Microbial populations												
Total protozoa, × 10^5/mL	12.1	10.6	13.3	11.3	14.6	11.9	13.2	11.3	0.17	< 0.01	< 0.01	0.16
Isotrichidae, × 10^3/mL	5.64^{c}	6.27^{c}	6.14^{c}	16.8^{b}	6.38^{c}	21.7^{a}	6.05	15.1	0.57	< 0.01	< 0.01	< 0.01
Ophryoscolecidae, × 10^5/mL	12.0	10.5	13.2	11.1	14.5	11.7	13.2	11.1	0.17	< 0.01	< 0.01	0.11
Dasytricha ruminantium, × 10^3/mL	5.10^{c}	5.29^{c}	5.21^{c}	15.5^{b}	5.24^{c}	19.8^{a}	5.18	13.6	0.53	< 0.01	< 0.01	< 0.01
Isotricha prostoma, × 10^3/mL	0.25^{d}	0.19^{d}	0.49^{c}	0.93^{b}	0.66^{c}	1.37^{a}	0.47	0.87	0.05	< 0.01	< 0.01	< 0.01
Isotricha intestinalis, × 10^3/mL	0.24	0.33	0.30	0.43	0.47	0.52	0.34	0.43	0.02	< 0.01	< 0.01	0.48
Entodinium spp., × 10^5/mL	11.9	10.4	13.0	11.1	14.3	11.6	13.1	11.0	0.17	< 0.01	< 0.01	0.12
Ostracodinium gracile, × 10^3/mL	7.16^{c}	5.79^{c}	9.69^{b}	6.64^{c}	12.8^{a}	9.60^{b}	10.0	7.20	0.24	< 0.01	< 0.01	< 0.01
Polyplastron multivesiculatum, 10^3/mL	1.15^{b}	0.70^{c}	2.24^{a}	0.91^{b}	2.45^{a}	1.23^{b}	1.93	0.94	0.07	< 0.01	< 0.01	< 0.01

[1] *NH_3* ammonia, *VFA* volatile fatty acid

[2] The ruminal fluid was obtained from each cannulated cow from three locations in the midventral sac of the rumen before morning feeding (0 h), 3 h after morning feeding, and 6 h after morning feeding

[3] *CON* control diet, *PLS* paulownia leaves silage diet

[4] *T* treatment, *H* hours

[a,b,c,d,e] Means with different superscript letters differ significantly ($P<0.05$) among the treatments and hours in a row

multivesiculatum ($P<0.01$) also were affected by treatment × time interaction. The first three protozoa mentioned were increased by PLS at 3 h and 6 h, but were similar at 0 h; whereas *Ostracodinium gracile* population was greater at 3 h and 6 h, and *P. multivesiculatum* was greater at 0, 3, and 6 h in the PLS diet than in the control diet. The inclusion of PLS in the diet decreased total protozoa, Ophryoscolecidae and *Entodinium* spp. counts ($P<0.01$).

Feeding PLS to cannulated cows increased ($P<0.05$) the populations of all bacterial species examined in this study, except *Ruminococcus flavefaciens*, *Ruminococcus albus* and *Lactobacillus* spp. (Table 5). The decreases in abundances of total archaea, Methanobacteriales and Methanomicrobiales were noted in the experimental group ($P<0.01$). The inclusion of PLS did not affect degradability of DM, OM, and NDF, but a lower crude protein degradability was observed ($P<0.01$) in PLS diet. The EE degradability increased due to PLS feeding ($P<0.05$). Lowered ($P<0.01$) methane production (g/d) and yield (g/kg DM intake) in PLS diet were noted compared to the CON diet; however, PLS diet did not alter CO_2 production in the rumen-cannulated cows.

In the Exp. 2 (cannulated cows), the ruminal fluid of PLS cows was characterized by altered proportions of selected FAs. The level of C8:0, C10:0, C12:0, C16:0, C16:1, C17:1 and C18:0 decreased ($P<0.05$; Table 6) whereas the level of C14:1, C:15:0, C:15:1, and C17:0 increased ($P<0.01$). Regarding the proportion of C8:0, C12:0, C14:1, C:15:0, C:15:1, C16:0, and C16:1, the treatment × time interaction was also observed ($P<0.05$). The proportion of C18:1 *trans*-10, C18:1 *trans*-11, C18:2 *cis*-9, *cis*-12, C18:2 *cis*-9, *trans*-11, and C18:2 *trans*-10, *cis*-12 increased in both treatment ($P<0.01$) and time-dependent ($P<0.01$) manners in PLS treatments. The PLS diet tended to decrease C18:1 *cis*-9 ($P<0.05$), but increase C18:3n-6 and C18:3 *cis*-9, *cis*-12, *cis*-15 ($P<0.01$) in treatment-dependent. Sum of unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), PUFA, n-6 FA and sum of n-3 FA

Table 5 The effect on replacing alfalfa silage with paulownia leaves silage (60 g/kg) on bacteria, methanogens, methane (CH_4) production and digestibility measured in rumen-cannulated cows ($n=4$) (Exp. 2)

Item	Treatments[a]		SEM	*P*-value[b]
	CON	PLS		
Microbial populations				
*Ruminococcus flavefaciens**	1.18	10.77	2.79	0.09
*Fibrobacter succinogenes**	0.13	0.32	0.04	< 0.01
*Streptococcus bovis**	1.07	4.61	0.51	< 0.01
Prevotella spp.*	3.81	12.91	0.90	< 0.01
*Butyrivibrio proteoclasticus**	2.19	9.97	1.93	0.04
*Ruminococcus albus**	0.36	0.39	0.45	0.74
*Butyrivibrio fibrisolvens**	0.36	2.82	0.51	0.01
Lactobacillus spp.*	0.64	0.58	0.04	0.46
*Megasphaera elsdenii**	3.98	16.64	1.82	< 0.01
Total bacteria, × 10^9/mL	7.15	6.86	0.20	0.51
Total archaea, × 10^8/mL	6.28	5.28	0.19	< 0.01
Methanobacteriales, × 10^8/mL	4.33	3.44	0.15	< 0.01
Methanomicrobiales, × 10^7/mL	3.82	3.21	0.13	< 0.01
Dry matter intake	23.2	22.9	0.08	0.07
Total-tract digestibility [c], g/kg DM				
DM	631	618	4.42	0.15
OM	660	654	5.34	0.61
NDF	497	514	10.4	0.46
CP	616	584	6.07	< 0.01
EE	696	747	11.1	0.02
CH_4, g/d	459	410	9.80	< 0.01
CH_4, g/kg DMI	22.1	19.5	0.29	< 0.01
CO_2, g/d	11,403	12,008	203	0.97
CO_2, g/kg DMI	504	511	7.38	0.74

[a] *CON* control diet, *PLS* paulownia leaves silage diet; the percentage means of how many percentages of alfalfa was replaced with paulowina silage

[b] The results are considered to be significantly different at $P \leq 0.05$

[c] *DM* dry matter, *OM* organic matter, *CP* crude protein, *EE* ether extract, *NDF* neutral detergent fiber

* Abundance (log_{10} number of copies of *rrs* gene/mL of rumen sample)

increased ($P<0.01$) in the experimental group, which resulted in lower sum of SFA ($P<0.01$). Time-dependent variation ($P<0.01$) was observed in all of the above parameters, in addition to the sums from MUFA. The inclusion of PLS increased the sum of *trans* C18:1, sum of medium-chain FA, n-6/n-3 FA ratio and PUFA/SFA ratio ($P \leq 0.05$). The treatment × time interaction was noticed for the following FAs: C18:1 *trans*-10, C18:2 *cis*-9, *cis*-12, C18:2 *cis*-9, *trans*-11, and C18:2 *trans*-10, *cis*-12 and sum of other FA, SFA, UFA, PUFA, n-6 FA, *trans* C18:1, n-6/n-3 FA ratio, and PUFA/SFA ratio ($P<0.05$).

In vivo experiment (using commercial dairy cows)

The inclusion of PLS in the diet of dairy cows affected milk composition and ruminal methane concentration (Table 7). The PLS diets decreased protein and lactose yield ($P<0.05$), however, it did not affect milk yield, energy corrected milk (ECM) and fat yield. The fat content was not affected by replacing alfalfa silage with PLS, but PLS diet decreased protein and lactose content ($P<0.05$). Milk urea concentration was increased ($P<0.05$) by PLS feeding. Methane concentration in the exhaled gas decreased ($P<0.01$) about 14% in the PLS group compared to the CON group.

The PLS diet increased proportions of C15:0, C16:1, C18:2 *cis*-9 *trans*-11, C18:3 *cis*-9 *cis*-12 *cis*-15 and C20:4 n-6 ($P<0.01$) (Table 8). The proportions of C18:1 *trans*-10, C18:1 *trans*-11, and the sum of *trans*-C18:1 was decreased ($P<0.05$) by PLS feeding to dairy cows. The PLS diet decreased the total SFA ($P<0.05$) proportion, but increased the total UFA ($P<0.05$) and PUFA ($P<0.05$) proportions. The PUFA/SFA ratio was higher ($P<0.05$) in the PLS group than in the CON group, but the n-6/n-3 FA ratio was lower ($P<0.01$) in the PLS group than in the CON group. Supplementation of PLS increased desaturase indices of C14:1, C16:1, and rumenic acid/(vaccenic acid + rumenic acid) ($P<0.05$). Furthermore, the PLS affected the relative transcript abundances of five out of six analyzed genes. Replacing alfalfa silage with PLS resulted in increased mRNA expressions of all genes ($P<0.01$) except *ACACA* gene (Fig. 1).

Discussion

The present study aimed at assessing the effect of replacing alfalfa silage with PLS in the dairy cow diet on ruminal fermentation processes such as VFA profile, methanogenesis, and biohydrogenation. We have demonstrated a beneficial effect of new forages PLS on ruminal fermentation processes and milk quality without a negative impact on milk production performance.

Dynamics of microorganism populations (bacteria, methanogens, protozoa) and ruminal fermentation characteristics (pH, ammonia, VFA, methanogenesis)

The dietary use of paulownia leaves has been studied for pigs, small ruminants, rabbits, and birds [6, 7] but no study has been conducted in high-producing dairy cows. The present study investigated ruminal fermentation, microbiota, milk production performance, and methane production variables. We recently published promising results of PLS on the ruminal environment [4]. The advantage of PLS over the fresh paulownia leaves resulted from high nutritive value (comparable to alfalfa) as well as high content of bioactive phenolic compounds (BAC),

Table 6 The effect on replacing alfalfa silage with paulownia leaves silage (60 g/kg) on fatty acid (FA) proportion (g/100 g of FA) in ruminal fluid ($n=4$) (Exp. 2)

Item	0 h		3 h		6 h		Treatment[1]		SEM[2]	*P*-value[3]		
	CON	PLS	CON	PLS	CON	PLS	CON	PLS		T	H	T × H
C8:0	0.13^{a}	0.092^{b}	0.11^{a}	0.089^{b}	0.088^{b}	0.11^{a}	0.11	0.10	0.006	0.02	0.21	< 0.01
C10:0	0.13	0.10	0.14	0.10	0.13	0.10	0.13	0.10	0.005	< 0.01	0.72	0.98
C12:0	0.44^{a}	0.10^{b}	0.45^{a}	0.10^{b}	0.38^{a}	0.10^{b}	0.42	0.10	0.019	< 0.01	0.02	0.04
C14:0	1.12	1.05	1.10	0.96	0.93	0.95	1.05	0.98	0.03	0.04	< 0.01	0.12
C14:1	1.18^{b}	1.92^{a}	1.14^{b}	1.89^{a}	1.16^{b}	1.58^{a}	1.16	1.80	0.05	< 0.01	< 0.01	< 0.01
C15:0	1.17^{b}	1.52^{a}	1.10^{b}	1.54^{a}	1.12^{b}	1.44^{a}	1.13	1.50	0.02	< 0.01	0.01	0.01
C15:1	0.46^{b}	0.77^{a}	0.47^{b}	0.73^{a}	0.49^{b}	0.66^{a}	0.47	0.73	0.02	< 0.01	0.01	< 0.01
C16:0	20.4^{a}	19.0^{b}	19.7^{b}	19.5^{b}	20.6^{a}	19.3^{b}	20.3	19.2	0.15	< 0.01	0.23	0.02
C16:1	0.48^{a}	0.22^{b}	0.46^{a}	0.22^{b}	0.32^{a}	0.23^{b}	0.42	0.22	0.002	< 0.01	0.01	< 0.01
C17:0	0.56	0.60	0.52	0.62	0.52	0.59	0.54	0.60	0.009	< 0.01	0.08	0.07
C17:1	0.12	0.079	0.10	0.10	0.12	0.087	0.11	0.087	0.005	< 0.01	0.95	0.10
C18:0	47.8	45.9	47.4	46.7	46.9	45.1	47.4	45.9	0.42	< 0.01	0.17	0.55
C18:1 *trans*-10	1.36^{b}	2.51^{a}	1.23^{b}	2.57^{a}	1.31^{b}	2.78^{a}	1.30	2.62	0.07	< 0.01	< 0.01	< 0.01
C18:1 *trans*-11	0.41	0.67	0.46	0.70	0.46	0.74	0.45	0.70	0.02	< 0.01	< 0.01	0.36
C18:1 *cis*-9	5.67	5.78	6.06	5.56	6.64	5.96	6.13	5.76	0.14	0.03	< 0.01	0.21
C18:2 *cis*-9 *cis*-12	5.27^{c}	7.38^{a}	6.52^{b}	6.78^{b}	6.74^{b}	7.47^{a}	6.14	7.19	0.14	< 0.01	< 0.01	< 0.01
C18:2 *cis*-9 *trans*-11	0.16^{b}	0.86^{a}	0.24^{b}	0.87^{a}	0.21^{b}	0.87^{a}	0.20	0.87	0.03	< 0.01	< 0.01	0.03
C18:2 *trans-10 cis*-12	0.12^{b}	0.16^{a}	0.15^{b}	0.14	0.15^{b}	0.17^{a}	0.14	0.16	0.005	< 0.01	< 0.01	< 0.01
C18:3 n-6	0.16	0.17	0.16	0.17	0.16	0.19	0.16	0.18	0.005	< 0.01	0.21	0.61
C18:3 *cis*-9 *cis*-12 *cis*-15	1.29	1.38	1.15	1.34	1.23	1.34	1.23	1.35	0.02	< 0.01	0.01	0.27
Sum of other FA[4]	9.07^{c}	9.83^{a}	9.80^{a}	9.42^{b}	9.48^{b}	9.90^{a}	9.44	9.71	0.12	0.08	0.36	< 0.01
Sum of SFA	74.5^{a}	69.4^{b}	73.0^{a}	70.5^{ab}	71.9^{a}	68.7^{b}	73.2	69.5	0.36	< 0.01	< 0.01	0.02
Sum of UFA	25.5^{c}	30.6^{a}	27.0^{b}	29.5^{ab}	28.1^{b}	31.3^{a}	26.8	30.5	0.36	< 0.01	< 0.01	0.02
Sum of MUFA	17.3	20.9	17.3	20.4	17.9	20.9	17.5	20.7	0.28	< 0.01	0.27	0.65
Sum of PUFA	8.30^{b}	9.73^{a}	9.47^{a}	8.98^{b}	9.56^{a}	10.1^{a}	9.08	9.59	0.15	< 0.01	< 0.01	< 0.01
Sum of n-6 FA	6.47^{c}	8.43^{a}	8.13^{ab}	7.85^{b}	8.33^{a}	8.98^{a}	7.60	8.41	0.17	< 0.01	< 0.01	< 0.01
Sum of n-3 FA	1.29	1.40	1.15	1.32	1.23	1.34	1.23	1.35	0.02	< 0.01	< 0.01	0.58
Sum of *trans* C18:1	2.38^{b}	4.99^{a}	2.33^{b}	5.03^{a}	2.37^{b}	5.34^{a}	2.36	5.11	0.14	< 0.01	< 0.01	< 0.01

Table 6 (continued)

Item	0 h		3 h		6 h		Treatment[1]		SEM[2]	*P*-value[3]		
	CON	PLS	CON	PLS	CON	PLS	CON	PLS		T	H	T × H
Sum of medium-chain FA	26.2	26.9	25.3	26.8	25.9	26.3	25.9	26.7	0.20	< 0.01	0.23	0.09
Sum of long-chain FA	73.6	73.0	74.0	73.0	73.8	73.5	73.8	73.1	0.21	0.01	0.46	0.45
n-6/n-3 FA ratio	5.13^{e}	5.57^{de}	7.80^{a}	6.05^{c}	6.65^{b}	6.69^{b}	6.44	6.09	0.19	0.05	< 0.01	< 0.01
PUFA/SFA ratio	0.11^{c}	0.14^{a}	0.13^{b}	0.13^{b}	0.13^{b}	0.15^{a}	0.13	0.14	0.003	< 0.01	< 0.01	< 0.01

[1] *CON* Control diet, *PLS* paulownia leaves silage diet

[2] *SEM* standard error of means for the main effect

[3] *T* treatment, *H* hour

[4] Other FA include C14:1 *iso,* C14:1 *anteiso,* C15:1 *anteiso,* C16:1 *anteiso*, C17:1 *anteiso,* C18:1 *trans*-6–8, C18:1 *trans*-9, C18:1 *cis*-11, C18:1 *cis*-12, C18:1 *cis*-13, C18:1 *cis*-14, C18:2 *trans*-11 *cis*-15, C20:0, C20:1 *trans,* C21:0, C18:3 *cis*-9 *trans* 11 *cis*-15, C22:0, C23:0, C24:0, and C24:1

[a-e] Means with different superscript letters differ significantly ($P < 0.05$) among the treatments and hours in a row

Table 7 The effect on replacing alfalfa silage with paulownia leaves silage (60 g/kg) on milk production performance and methane concentration of commercial dairy cows ($n = 16$) (Exp. 3)

Item	Treatments[a]		SEM	*P*-value[b]
	CON	PLS		
DM intake	23.5	22.9	0.16	0.11
Milk yield				
Milk, kg/d	33.9	32.5	0.46	0.08
ECM[c], kg/d	35.5	33.8	0.67	0.17
Fat, g/d	1349	1201	68.2	0.23
Protein, g/d	896	831	16.5	0.02
Lactose, g/d	1296	1189	23.5	0.01
Milk composition				
Fat, g/kg	43.8	44.0	0.99	0.91
Protein, g/kg	34.1	33.4	0.16	0.03
Lactose, g/kg	49.3	47.9	0.28	0.01
Urea, mg/L	224	249	3.06	0.02
Methane, µg/L	211	185	7.1	< 0.01

[a] *CON* Control diet, *PLS* Paulownia leaves silage diet

[b] The results are considered to be significantly different at $P \leq 0.05$

[c] Energy corrected milk calculated according the following equation: ECM = milk yield (kg) × (38.3 × fat (g/kg) + 24.2 × protein (g/kg) + 783.2)/3.140 [23]

including phenolic acid (47 g/kg DM) and flavonoid (13 g/kg DM) contents [4]. The influence of PLS on ruminal fermentation characteristics was likely due to the high content of BAC, such as phenolic acids and flavonoids. The present in vitro and in vivo study is a direct continuation of previous work [4]. The intake of phenolic acid and flavonoid in case of the highest PLS dose (60 g/kg DM) was 65 g/d/cow and 18 g/d/cow, respectively. Dietary BAC can exert modulatory or even antimicrobial effects on ruminal microbial populations and thus the basic ruminal characteristics [24, 25]. Therefore, higher ruminal pH ($P < 0.01$) of PLS diets can be partly explained by BAC activity, which decreased ($P < 0.01$) the acetate-to-propionate ratio due to an increase of the numbers of lactate-consuming and propionate- producing bacteria preventing ruminal pH reduction [26]. In the current study, although population of *Streptococcus bovis* increased in the PLS groups ($P < 0.01$), its growth may not cause lactic acid accumulation in the ruminal fluid and thus pH reduction [27]. Increased pH was probably linked to a higher abundance of *Megasphaera elsdenii* ($P < 0.01$) in the PLS diets as noted both in the present in vitro and in vivo experiments. *M. elsdenii* utilizes lactic acid as the main source of energy and thus can protect other ruminal microorganisms from the negative impact of low pH resulting from lactic acid accumulation [28]. High pH positively affects the adhesion of fibrolytic bacteria to the feed particles and thus their degradation [29]. This phenomenon was

Table 8 The effect of replacing alfalfa silage with paulownia leaves silage (60 g/kg) on milk fatty acids (FA) composition (g/100 g FA) and desaturation (DI) of milk of dairy cows ($n = 16$) (Exp. 3)

Item[a]	Treatment[b]		SEM	*P*-value[c]
	CON	PLS		
Saturated FA				
C8:0	0.94	0.92	0.01	0.41
C10:0	2.86	2.91	0.08	0.78
C12:0	3.80	3.75	0.07	0.76
C14:0	11.8	11.5	0.13	0.22
C15:0	1.46	1.59	0.02	< 0.01
C16:0	34.3	33.1	0.44	0.16
C18:0	11.5	11.6	0.13	0.65
Monounsaturated FA				
C16:1	1.33	1.74	0.05	< 0.01
C18:1 *trans*-10	0.53	0.44	0.01	< 0.01
C18:1 *trans*-11	0.60	0.51	0.02	0.02
C18:1 *cis*-9	21.3	22.6	0.32	0.06
Polyunsaturated FA				
C18:2 *cis*-9 *cis*-12	3.23	3.16	0.03	0.38
C18:2 *cis*-9 *trans*-11	0.56	0.72	0.01	< 0.01
C18:2 *trans*-10 *cis*-12	0.11	0.12	0.003	0.13
C18:3 *cis*-9 *cis*-12 *cis*-15	0.39	0.47	0.01	< 0.01
C20:4 n-6	0.13	0.16	0.002	< 0.01
C20:5 n-3	0.058	0.062	0.004	0.30
C22:6 n-3	0.051	0.057	0.002	0.19
Other FA[c]	4.91	4.73	0.06	0.56
Total FA				
Sum of SFA	67.9	66.2	0.39	0.04
Sum of UFA	32.1	33.8	0.39	0.04
Sum of MUFA	27.4	28.8	0.35	0.05
Sum of PUFA	4.66	4.90	0.05	0.03
Sum of n-6 FA	3.98	3.92	0.05	0.51
Sum of n-3 FA	0.51	0.60	0.01	< 0.01
Sum of *trans*-C18:1	1.69	1.53	0.02	< 0.01
Sum of MCFA	52.9	51.6	0.47	0.16
Sum of LCFA	43.2	44.6	0.47	0.18
PUFA/SFA ratio	0.07	0.08	0.001	0.02
n-6/n-3 FA ratio	7.89	6.76	0.12	< 0.01
DI C14:1/(C14:0 + C14:1)[d]	0.28	0.30	0.004	0.01
DI C16:1/(C16:0 + C16:1)	0.037	0.051	0.002	0.02
DI C18:1/(18:0 + C18:1)	0.65	0.66	0.003	0.06
DI RA/(VA + RA)	0.48	0.59	0.01	< 0.01

[a] *SFA* saturated fatty acids, *UFA* unsaturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA*, polyunsaturated fatty acids, *MCFA* medium-chain fatty acids, *LCFA* long-chain fatty acids, *RA* rumenic acid (C18:2 *cis*-9, *trans*-11), *VA* vaccenic acid (C18:1 *trans*-11)

[a] *CON* control diet, *PLS* paulownia leaves silage diet

[b] The results are considered to be significantly different at $P \leq 0.05$

[c] Other FA include C14:1, C15:1. C17:0, C17:1, C18:1 *trans*-6–8, C18:1 *trans*-9, C18:1 *cis*-11, C18:1 *cis*-12, C18:1 *cis*-13, C18:1 *cis*-14, C19:0, C20:0, C20:1 *trans,* C18:3 n-6, C21:0, C20:2, C22:0, C20:3 n-6, C22:1 n-9, C20:3 n-3, C23:0, C22:2, C24:0 and C24:1

[d] DI C14:1, DI C16:1, DI C18:1, DI RA/(VA + RA) were calculated according to Bryszak et al. [20]

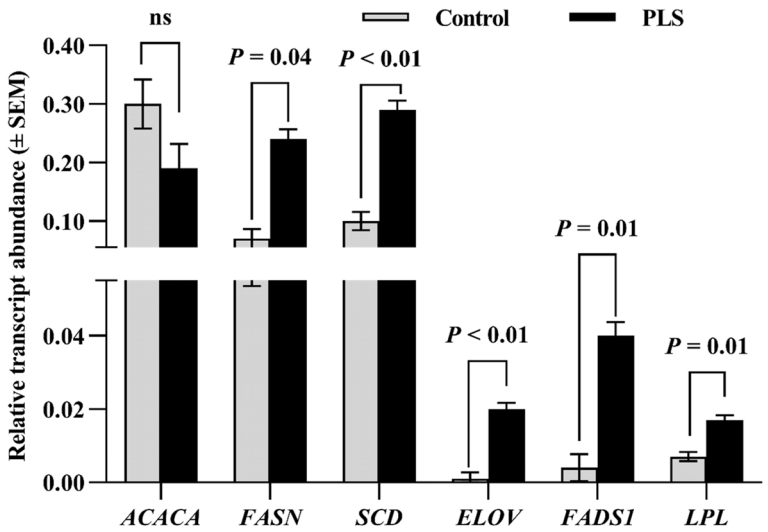


Fig. 1 The effect of replacing diet with paulownia leaves silage (PLS: 60 g/kg) on expression of acetyl-CoA carboxylase 1 (*ACACA*), fatty acid synthase (*FASN*), stearoyl-CoA desaturase (*SCD*), fatty acid elongase (*ELOVL*), fatty acid desaturase 1 (*FADS1*), and lipoprotein lipase (*LPL*) genes in the milk of lactating cows (ns—not significant). (Exp. 3)

confirmed in the present study which demonstrated an increase ($P<0.01$) in the population of some bacteria species such as *Butyrivibrio fibrisolvens* and *Prevotella* spp. in response to PLS supplementation. Zhan et al. [30] showed that flavonoids (from 20 to 100 mg/kg body weight) increased *B. fibrisolvens* population and feed digestion through changes in the population of ruminal microorganisms. In the present experiment on cannulated cows, flavonoids were supplemented at the rate of 29 mg/kg of body weight, which corresponded to the lower range of Zhan et al. [30] study.

Besides changes in pH, PLS diet increased ammonia concentration ($P<0.01$), both in vitro and in vivo. This observation can be explained by higher ruminal CP degradation compared to the control with non-synchronization of available energy and ammonia in the ruminal fluid [4, 31]. On the other hand, the CP total-tract degradability was reduced ($P<0.01$) in the PLS cows. Such difference may result from different degradation of protein or distinct solubility of feed protein from alfalfa and paulownia leaves silages [4]. Changes in the observed ammonia concentration may also be associated with alterations in the number of protozoa and bacteria. Although lower protozoa can decrease ammonia concentration due to reduced recycling of engulfed bacterial protein by protozoa, decreased protozoal number may increase bacterial activity or growth in the rumen. In the current study, Ophryoscolecidae decreased ($P<0.01$), whereas Isotrichidae protozoa significantly increased ($P<0.01$) in the PLS diet. A previous study indicated that Ophryoscolecidae protozoa have higher bacterivory activity [32]. Thus, a decrease in the number of Ophryoscolecidae could increase selected bacterial populations. In the current study, numbers of proteolytic bacteria such as *Prevotella* spp. ($P<0.01$) and *B. fibrisolvens* ($P<0.05$) in PLS diets increased considerably, both in vitro and in vivo. *Prevotella* spp. in the rumen are the predominant proteolytic bacteria with diverse and broad range of peptidase activities and represent 20% to 60% of the bacterial abundance [33]. Therefore, it seems that the PLS diet increased proteolytic activity in the rumen, resulting in greater ammonia concentration in the rumen.

Inhibition of ruminal methanogenesis is usually associated with increase in propionate concentration due to competition for hydrogen. High levels of metabolic hydrogen in the rumen may shift fermentation towards propionate by *Prevotella* spp. that increased ($P<0.01$) in the PLS diets. Ruminal *Prevotella* species use different pathways for propionate production utilizing metabolic hydrogen via succinate or acrylate pathways for fermentation of sugars and lactate [34]. Moreover, Seradj et al. [35] suggested that elevated propionate can result from flavonoid supplementation (0.2 mg/g DM), which reduced methane production ($P<0.01$) and population of methanogenic archaea ($P<0.01$), and also increased ($P<0.01$) population of *M. elsdenii* which has been confirmed in the present study.

In our previous in vitro study, paulownia leaves with high total polyphenols content (31 or 35 g/kg DM) lowered ruminal methane production and methanogen populations without affecting substrate degradability and volatile fatty acid concentrations [8]. Similar results were also noted in the current experiments. Besides, the addition of flavonoid-rich plants may impair the growth of some protozoa-associated methanogens [36]. It has been estimated that protozoa-associated methanogens

are responsible for up to 37% of methane production [37]. In the present study, results obtained from rumen-cannulated dairy cows verified those from the in vitro experiment. This included reduced methane emissions ($P < 0.01$), increased pH ($P < 0.01$) and ammonia concentration ($P < 0.01$), elevated propionate and butyrate concentrations ($P < 0.05$), decreased methanogens ($P < 0.01$) and Ophryoscolecidae numbers ($P < 0.01$), and increased Isotrichidae population ($P < 0.01$). Differences in protozoa behavior in the ruminal fluid from rumen-cannulated cows (Exp. 2) may suggest their distinct reaction to the BAC of PLS origin.

Another effect of BAC x microorganisms' interaction is the increase ($P < 0.01$) in the population of *Fibrobacter succinogenes*. The increase in *F. succinogenes* and *B. fibrisolvens* populations might be due to the presence of phenolic acids in PLS. Some phenolic acids such as hydroxycinnamic acid, syringic acid, and p-hydroxybenzoic acid were found to stimulate specific bacterial populations in a dose-dependent manner [38]. Though high concentrations of polyphenols are toxic to ruminal bacteria, a low concentration of polyphenol extract was also found to stimulate the *F. succinogenes* in the rumen [39]. According to Mitsumori et al. [34], elevated numbers of *F. succinogenes* known as non-H_2 producers may mitigate methane production through H_2 restriction. Zhang et al. [40] reported that increased propionate and less acetate negatively affect the abundance of *F. succinogenes* and *B. fibrisolvens*. We did not observe such relations regarding *F. succinogenes* and *B. fibrisolvens* population but we noticed decreased acetate ($P < 0.01$) and increased propionate concentration ($P < 0.01$) in ruminal fluid in in vitro and in vivo studies.

Dynamics in bacteria populations, rumen (FA) and milk parameters (basic composition, FA, transcript expression)

The present study revealed a tendency of reduction in milk production and a decrease in protein and lactose yield ($P < 0.05$) in response to PLS treatment. Khorsandi et al. [1] also reported decreased milk protein and lactose yield after using 120 g/kg DM of pomegranate by-product silage, rich in phenolic compounds (49 g/kg DM). The inclusion of forages rich in phenolic compounds can decrease CP digestibility, as was observed in the current studies, and therefore may negatively affect CP ruminal and post-ruminal digestion. Moreover, in our previous study, the rapidly soluble fraction of protein degradation was higher in PLS than in alfalfa silage, which indicates an imbalance of available energy deficit accompanying increased ammonia concentration in the ruminal fluid [4]. Higher ammonia levels in the ruminal fluid may interact with milk parameters and result in increased urea content ($P < 0.05$) in milk when the diet was supplemented with PLE [41]. Considering this, it may be important to balance the dairy cow rations for easily fermentable nonstructural carbohydrates such as concentrates.

With regard to the fatty acids, an increase in C15:0 proportion ($P < 0.01$) in the ruminal fluid and milk suggests higher activity of *F. succinogenes.* These microorganisms are responsible for synthesis of some FAs, including odd chain (mainly C15:0) and branched-chain FAs [42]. The PLS diet also modulated biohydrogenation of long chain FA such as C18:2 *cis*-9, *cis*-12. According to Szczechowiak et al. [9], BAC derived from lingonberry (*Vaccinium vitis-idaea*) affected the biohydrogenation process with an increase in CLA isomer (C18:2 *cis*-9, *trans*-11) and vaccenic acid (C18:1 *trans*-11) as intermediates, which was also observed in the current study. The amount of C18:2 *trans*-10, *cis*-12 in the ruminal fluid may be influenced by the activity of *M. elsdenii* as shown by Kim et al. [43]. The amount of this conjugated linoleic acid isomer (CLA), and also α-linolenic acid is usually low in milk [44]. However, we did not observe similar changes in the milk.

In case of two FAs, markers of the biohydrogenation process such as C18:1 *trans*-11 and C18:2 *cis*-9 *trans*-11, parallel changes were noticed in ruminal fluid and in milk. Synthesis of these two isomers is dependent on the activity of *B. fibrisolvens*, one of the dominant group A bacteria in ruminal biohydrogenation responsible for transforming UFA into SFA [39, 45]. Despite an increase in abundance of *B. fibrisolvens* in ruminal fluid of the PLS group, no elevation in SFA in favor of UFA was noticed in the current study. This may be due to the activity of Δ9-desaturase affecting proportion of the C18:1 *trans*-11 [20]. On the other hand, BAC may reduce Δ9-desaturase activity, which may lead to the reduction of the C18:1 *trans*-11 in the milk [46]. Another factor affecting concentration of the C18:1 *trans*-11 is the activity of the *B. proteoclasticus,* a B bacteria group capable of efficiently biohydrogenating PUFA to SFA [47]. *B. proteoclasticus* is recognized as an important bacterium that converts C18:1 *trans*-11 to C18:0 and is one of the most sensitive ruminal bacteria to changes in dietary PUFA [48, 49]. Despite an increase in the population of *B. proteoclasticus* in response to PLS, the proportion of C18:1 *trans*-11 did not reduce. Elevated PUFA content was also not related to the reduction of this bacterial species. Considering the complexity of ruminal processes, some other factors cannot be ruled out.

Milk quality in terms of FA composition also depends on the activity of genes being expressed in the mammary gland. We investigated the transcript expression of six genes controlling FA metabolism. The relative transcript abundance of 5 genes increased in response to PLS. The fatty acid synthase (FASN), a multifunctional

protein, is responsible for de novo biosynthesis of long-chain PUFAs [50]. An increase in the *FASN* transcript level was not accompanied by an elevation in long-chain FAs although the total and n-3 PUFA content increased, thus improving milk quality. Another factor affecting the elevation of the mentioned PUFAs may be the higher transcript level of the *ELOVL5* gene. This gene encodes a protein responsible for extending long-chain PUFA [51, 52]. According to those authors, an increase in mRNA level of *ELOVL5* gene elevated both n-6 and n-3 PUFA content in milk. Lack of the *ELOVL5* effect on the n-6 PUFA can be linked to increased transcript abundance of *FADS1,* a gene limiting the n-3/n-6 rate of PUFA synthesis [53]. In our study, a major increase was observed for n-3 FAs (e.g., C18:3 *cis*-9, *cis*-12, *cis*-15) whereas the proportion of only C20:4 n-6 increased without changing the total n-6 PUFA. We did not observe a limitation of the C18:2 *trans*-10, *cis*-12 proportion although the increased abundance of the *LPL* mRNA was noticed. Unlike in the present study, Bryszak et al. [54] described the decreased level of the *LPL* transcript accompanied by the reduced proportion of this FA. Milk quality is also influenced by MUFA content, especially C18:1 *cis*-9, which is influenced by the *SCD* gene-regulating de novo synthesis of endogenous FAs [55]. Despite a higher transcript content of *SCD* gene in the PLS group, only an increasing trend of C18:1 *cis*-9 proportion was observed for PLS diet.

Conclusions

Under the conditions of the present study, PLS (60 g/kg DM) reduced methanogenesis, beneficially modulated ruminal fermentation and biohydrogenation processes without a negative impact on milk production performance of lactating dairy cows. Dietary PLS also improved milk FA profile including greater proportions of total UFA, PUFA, conjugated linoleic acid, and C18:1 *trans*-11 along with reduction of n6/n3 ratio. The only negative impact of PLS was an increased ruminal ammonia concentration affecting milk urea content. For this reason, a better energy and protein synergy in PLS-containing diets would be required.

Abbreviations

aNDF: Ash free NDF; A/P ratio: Acetate/Propionate ratio; BAC: Biologically active compounds; BH: Biohydrogenation; CH_4: Methane emission; CO_2: Carbon dioxide emission; CLA: Conjugated linoleic acid; CP: Crude protein; DΔ: Desaturation Δ at –*n*; DI: Desaturation index; DM: Dry matter; DMD: Dry matter digestibility; DMI: Dry matter intake; DNA: Deoxyribonucleic acids; EE: Ether extract; EI: Elongase index; ELOVL5: Fatty acid elongase 5; FA: Fatty acids; FADS1: Fatty acid desaturase 1; FASN: Fatty acid synthase; FISH: Fluorescence in situ hybridization; IVDMD: In vitro dry matter digestibility; LA: *α*-Linoleic acids; LCFA: Long chain fatty acids; LT: Longissimus thoracis; LNA: *α*-Linolenic acids; LPL: Lipoprotein lipase; MCFA: Medium chain fatty acids; mRNA: Messenger-RNA; MUFA: Monounsaturated fatty acids; NDFD: Neutral detergent fibre digestibility; NH_3: Ammonia; OM: Organic matter; PBS: Phosphate-buffered saline; paulownia leaves silage; PLS60: Paulownia leaaves silege; Diets containing 60 g/kg DM of PLS; PUFA: Polyunsaturated fatty acids; qPCR: Quantitative PCR; RA: Rumenic acid; RT: Transcription reaction; Rusitec: Rumen simulation technique; SCD: Stearoyl-CoAdesaturase; SFA: Saturated fatty acids; SPE: Solid phase extraction; TI: Thrombogenicity; UFA: Unsaturated fatty acids; VA: Vaccenic acid; VFA: Volatile fatty acids.

Supplementary Information

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Additional file 1: Table S1. The sequences of primers specific to the analyzed bacteria species.

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Authors' contributions

HH designed the study protocol, provided laboratory analysis, interpreted the data and wrote first version of the manuscript. DL reviewed and improved the manuscript. MSz improved the manuscript. AKP interpreted the data and reviewed the manuscript. MK provided laboratory analysis of methanogens. PK provided laboratory analysis of bacteria. MG provided statistical analyses. PK performed the laboratory analysis. SS provided laboratory analysis. DP provided laboratory analysis of rumen fluid. AC designed the study protocol, interpreted the data, wrote the manuscript, and finally reviewed the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

Data available by the Author by request (adam.cieslak@up.poznan.pl).

Declarations

Ethics approval and consent to participate

The present study consisted of three consecutive experiments: one in vitro (Rusitec system) and two in vivo (rumen cannulated cows in lactation and milking cows under commercial production system). All experimental procedures were performed according to the National Ethical Commission for Animal Research guidelines (Ministry of Science and Higher Education, Poland). The study was approved by the Local Ethics Commission (decision no. 14/2019).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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