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Effects of saponins from fresh and ensiled alfalfa (*Medicago sativa L.*) on enteric methane emission and biohydrogenation in dairy cows

Świeża i zakiszona lucerna siewna (*Medicago sativa L.*) jako źródło saponin oddziaływujących na poziom produkcji metanu oraz proces biouwodorowania u krów mlecznych

Summary of the doctoral dissertation

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Summary

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Among the sources of anthropogenic origin, ruminant animals kept under production conditions are the leading producers of greenhouse gases, including methane. Animal production must meet the growing demand for food while limiting the negative impact on the environment. Methane is one of the greenhouse gases primarily responsible for climate change. It is formed in the rumen by the action of microorganisms during fermentation processes. For many years, dietary components have been searched for, which, when used in the ruminants' diets, mainly dairy cows, will affect the population of microorganisms living in the rumen and, as a result, reduce the amount of methane produced. Because of the ban on the use of antibiotic growth promoters in animal nutrition in the European Union, feed components containing biologically active compounds affecting ruminal microorganisms, e.g., secondary plant metabolites, are sought. Alfalfa (*Medicago sativa L.*) is a popular legume grown widely in temperate climates. Alfalfa is a rich source of plant secondary metabolites, primarily saponins, with the potential to modulate rumen fermentation while reducing methane production and ammonia concentration by decreasing the population of rumen microbes, mainly protozoa.

As part of the research, an analysis of the content and profile of saponins was carried out in ten varieties of fresh and ensiled alfalfa. The structural changes to which saponins are subjected during the ensilage process were also determined using UPLC. Two varieties of alfalfa with the highest content of saponins were selected for further *in vitro* and *in vivo* studies. The in vitro studies consisted of three experiments performed using two short-term techniques for rumen fluid fermentation: batch culture and Hohenheim gas test, and the long-term technique, the so-called artificial rumen (RUmen SImulation

TEChnique; RUSITEC). *In vivo* studies were also carried out with cannulated dairy cows and dairy cows under commercial farm conditions. In the collected rumen fluid samples (*in vitro* and *in vivo*), the parameters characterizing the fermentation processes were determined; moreover, in the in vivo tests, the fatty acids profile in the rumen fluid and milk, and the basic parameters of milk, were determined.

As a result of the research, it was found that the ensiling process increases the concentration of saponins threefold and changes their profile in ensiled alfalfa compared to fresh material. Moreover, it has been confirmed that saponins, due to their antiprotozoal, antimethanogenic and antibacterial properties, modulate rumen processes, causing changes in the volatile fatty acid composition and fatty acid profile and reducing methane production. Additionally, saponins show different effectiveness depending on their source. The addition of alfalfa silage to the diet for dairy cows has a positive effect on the basic parameters of milk and the milk fatty acids profile. It has been shown that the most promising results were obtained with the use of Kometa alfalfa silage in the diet. To conclude, the Kometa alfalfa silage as a source of saponins can be successfully used as a valuable component of dairy cows' diets, reducing methane emissions.

Streszczenie

Świeża i zakiszona lucerna siewna (*Medicago sativa L.*) jako źródło saponin oddziaływujących na poziom produkcji metanu oraz proces biouwodorowania u krów mlecznych

Zwierzęta przeżuwające utrzymywane w warunkach produkcyjnych są, wśród źródeł pochodzenia antropogenicznego, głównymi producentami gazów cieplarnianych, w tym metanu. Metan jest jednym z gazów cieplarnianych, w dużej mierze odpowiedzialnym za zmiany klimatyczne. Powstaje w żwaczu w wyniku działania mikroorganizmów podczas procesów fermentacji. Od wielu lat poszukiwane są komponenty paszowe, które zastosowane w dawce pokarmowej dla zwierząt przeżuwających, głównie krów mlecznych wpłyną na populacje mikroorganizmów bytujących w żwaczu i w efekcie ograniczą ilość produkowanego metanu. Tym samym proces produkcji surowców pochodzenia zwierzęcego musi cechować się ograniczonym negatywnym wpływem na środowisko naturalne uwzględniając rosnące zapotrzebowanie. Wobec zakazu stosowania antybiotykowych stymulatorów wzrostu w żywieniu zwierząt na terenie Unii Europejskiej, poszukuje się komponentów paszowych zawierających związki biologicznie aktywne oddziałujące na mikroorganizmy żwacza, np. wtórne metabolity roślinne. Lucerna siewna (*Medicago sativa L.*), to popularna roślina bobowata, którą można powszechnie uprawiać w klimacie umiarkowanym. Lucerna jest bogatym źródłem wtórnych metabolitów roślinnych, przede wszystkim saponin. Saponiny przy umiarkowanych koncentracjach mogą wykazać potencjał do modulowania fermentacji w żwaczu przy jednoczesnym ograniczeniu produkcji metanu i stężenia amoniaku, poprzez redukcję populacji drobnoustrojów w żwaczu, głównie pierwotniaków.

W ramach przeprowadzonych badań wykonano analizę zawartości i profilu saponin w dziesięciu odmianach świeżej i zakiszonej lucerny. Określono również, przy użyciu UPLC,

zmiany strukturalne, którym poddawane są saponiny podczas procesu zakiszania. Do dalszych badań w warunkach *in vitro* i *in vivo* wybrano dwie odmiany lucerny (Verko i Kometa) o najwyższej zawartości saponin. Badania *in vitro* obejmowały trzy eksperymenty wykonane przy użyciu dwóch krótkoterminowych technik fermentacji płynu żwacza: batch culture i Hohenheim gas test oraz z wykorzystaniem techniki długoterminowej, tzw. sztucznego żwacza (RUmen SImulation TEChnique; RUSITEC). Przeprowadzono również badania w warunkach *in vivo* z udziałem krów mlecznych z założonymi przetokami żwaczowymi oraz w warunkach produkcyjnych. W pobranych próbach płynu żwacza (in vitro i in vivo) określono wskaźniki charakteryzujące procesy fermentacji, ponadto w badaniach *in vivo,* określono profil kwasów tłuszczowych w płynie żwacza i mleku a także podstawowe parametry mleka.

W efekcie przeprowadzonych badań stwierdzono, że proces kiszenia zwiększa trzykrotnie stężenie saponin oraz zmienia ich profil w zakiszonej lucernie w porównaniu ze świeżym materiałem. Ponadto, potwierdzono, że saponiny ze względu na swoje właściwości przeciwpierwotniacze, antymetanogenne i przeciwbakteryjne modulują procesy zachodzące w żwaczu, powodując zmiany w składzie lotnych kwasów tłuszczowych, profilu kwasów tłuszczowych oraz zmniejszają produkcję metanu. Dodatkowo, saponiny wykazują różną efektywność w zależności od ich źródła. Dodanie do dawki pokarmowej dla krów mlecznych kiszonki z lucerny wpływa korzystnie na podstawowe parametry mleka oraz profil kwasów tłuszczowych w mleku. Wykazano, że najbardziej obiecujące wyniki uzyskano stosując w dawce pokarmowej kiszonkę z lucerny odmiany Kometa. Podsumowując, kiszonka z lucerny odmiany Kometa jako źródło saponin może być z powodzeniem stosowana jako wartościowy składnik dawek pokarmowych dla krów mlecznych, który dodatkowo ogranicza emisję metanu.

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Małgorzata Szumacher	16%	conceptualization, methodology, writing - original draft
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Martyna Kozłowska	15%	participation in the conceptualization and creation of methodology, chemical composition analysis, collecting a literature database, writing part of the discussion on saponins and basic nutrients correlation, adjusting the manuscript to the journal's requirements,
Dawid Kuźnicki	3%	data curation,
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Mohamed El-Sherbiny	3%	methodology, writing - original draft,
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1. Introduction

Animal production faces a significant challenge to meet the growing demand for food while reducing emissions of harmful greenhouse gases. Reducing the emission of methane, carbon dioxide, and other pollutions is a severe challenge in the dairy industry due to society's growing expectations. There is also a need to provide adequate nutrition for animals, taking into account the deficiency of protein-rich dietary materials [1]. Ruminants are considered the primary greenhouse gas producers, with the amount estimated to be around 80% of the total emissions from enteric fermentation and about 14.5% of total anthropogenic greenhouse gas emissions [2]. For this reason, it is essential to maximize the performance of dairy cows and use resources responsibly [3].

Methane is produced by *Archaea* mainly with hydrogen and carbon dioxide in the process of methanogenesis. Methane is one of the leading greenhouse gases responsible for planetary heating and is causing climate change [4]. The mitigation of methanogenesis is also beneficial for increasing the dietary energy efficiency used in dairy cows' nutrition [5]. For this reason, the mitigation of methane emissions has gained scientific attention over the past few decades due to its adverse effects on the environment and animal production [6]. These mitigation strategies include modulation of rumen fermentation through various means of modification of rumen microbial diversity [7]. Dietary manipulation is directly related to changes in the rumen fermentation pattern and types of end products. Previously, antibiotics were used extensively as a dietary supplement for ruminants to modulate rumen fermentation and reduce methane emissions. In 2003, the European Commission banned the use of chemical supplements in the nutrition of ruminants (Directive 1831/2003 / CEE) because the excessive use of antibiotics leads to developing bacteria drug resistance, endangering human health [8]. Since then, animal nutrition scientists have focused on using natural products such as secondary plant metabolites, bioactive compounds of plants origin as an environmentally safe alternative

to synthetic chemicals in the ruminant feed ration [9]. Plants and their plant extracts have been extensively studied for their content of precursors and their influence on rumen fermentation through changes in ruminal microbial populations [10].

Alfalfa (*Medicago sativa L.*), also known as lucerne, is a popular legume plant commonly cultivated in a temperate climate, also in Poland. It is characterized by high nutritive value, reasonable protein quality, and high protein content, making it a suitable plant for use in animal diets, including high-producing dairy cows [11,12]. Due to its high protein content is an excellent pasture for ruminants, which can be used as green alfalfa grazing feed or as hay or pellets. However, in moist climates where field drying is difficult, alfalfa is possessed into silage instead of drying fresh material [13]. The process is quite complex in the case of alfalfa due to the low carbohydrate content and the risk of degrading the crude protein to destructible non-protein nitrogen during ensiling [14,15]. Furthermore, alfalfa is rich in secondary metabolites, especially saponins.

The saponins in alfalfa are pentacyclic triterpenes. These compounds occur in the form of glycosides of several aglycons, including medicagenic acid, zanhic acid, soyasapogenol, and hederagenin [16, 17]. Saponins can exist in three forms depending on the number of sugar chains attached to the aglycone: monodesmosidic, bisdesmosidic, or tridesmosidic form. The biological activity of saponins relies on the aglycone structure and the length and composition of carbohydrate side chains [18]. Saponins are characterized by foaming, hemolytic and antimicrobial properties, irritating the throat and modulating the permeability of the intestinal membrane. Saponins have antiprotozoal properties and are indirectly antimethanogenic because about 25% of methanogens are associated with protozoa in the rumen [19, 20]. The most active alfalfa saponins are medicagenic acid glycosides, and monodesmosides are more active than bisdesmosides. On the other hand, zanhic acid glycosides show little activity in this respect, while soyasapogenol glycosides are inactive [17]. Earlier

studies have shown that alfalfa saponins are biologically active and modulate ruminal fermentation [21]. To understand the interactions between saponins and ruminal microbial populations, knowledge of the relationship between chemical structure and activity of saponins is required. It was noted that plant materials used in animal nutrition should be assessed in terms of the content of saponins due to the wide variety of structures and actions of saponins. It is especially worth paying attention to forages produced by the compaction of plant material, such as the ensilage process, because in such cases, significant increases in the concentration of saponin and other anti-nutritional components may occur.

For this reason, the first step in the study of alfalfa saponins was to investigate the changes in the level and composition of saponins during fermentation. The results allowed us to select two alfalfa cultivars, Kometa and Verko, for further experiments due to the highest concentration of saponins. Then, the influence of total and individual saponins on the methane production and total microbial populations in rumen fluid was examined. The effect of the 2- fold and 4-fold increased dietary concentration of saponins on the rumen parameters was also investigated. These studies allowed us to conclude that saponins have differing effects depending on their sources (aerial and root parts of plants) and concentration. Alfalfa root saponins were shown to have the most visible effects. It has been found that all mixes of individual saponins (aerial, root, and all individual saponins) have a mitigating effect on methane production. The results of the total of all individual saponins did not differ much from the results of the mix with the root saponins only. For this reason, it was decided that the research using the artificial rumen, the RUSITEC system, would be carried out using, as previously mentioned, due to the highest concentration of saponins, Kometa and Verko alfalfa silages. In this case, it was also decided to use a saponin extract to verify whether a 4- fold increase in saponin content in the diet will significantly affect the results. However, in *in vivo* studies, after analyzing the data from the RUSITEC system experiments,

it was decided only to compare the alfalfa silage from the Kometa and Verko cultivars without the addition of saponin extracts.

Every year, the enthusiasm for producing health-promoting, enriched animal products grows. In the case of ruminants, the main focus was put on milk. Interest in milk, which contains increased amounts of acids such as cis-9, trans-11 C18:2 (conjugated linoleic acid; CLA), and 18:3 n-3, is constantly growing. For this reason, plants that replace the primary compounds in the diet of ruminants aimed at reducing methane emissions are also tested for their effects on milk and meat. It has been shown that, in addition to limiting the action on microbiota in the rumen, secondary plant metabolites, including saponins, also could improve milk nutritional quality by enhancing the content of fatty acids [22,23,24]. The research has shown that saponins derived from *Yucca schidigera* fed to dairy cows reduced the content of milk saturated fatty acids such as C6:0, C8:0 [25]. Milk from cows fed a diet supplemented with *Saponaria officinalis* root, rich in triterpenoid and saponins, had a higher content of cis-9 18:1, trans- 11 C18:1, cis-9, cis-12 C18:2 and cis-9, trans-11 CLA [26]. These showed that alfalfa containing high levels of saponin in a dairy cow's diet could positively modify the fatty acid composition in milk. In addition, replacing grass with legumes may result in higher dry matter consumption, increased milk yield, and increased milk fat and protein content due to a higher dietary protein content [27, 28, 29]. For this reason, the last experiment was carried out to investigate the effect of the addition of alfalfa silage containing saponins to the diet of high-producing dairy cows on the basic milk parameters and the composition of milk fatty acids. The composition of fatty acids in the ruminal fluid collected from cannulated cows was also analyzed to verify the rumen biohydrogenation process and the transformation of fatty acids in the rumen and milk.

2. Hypotheses:

- there is a higher content of saponins in ensiled alfalfa than in fresh material due to the processes taking place during ensiling,
- the process of ensiling does not significantly affect the nutrient composition of alfalfa,
- in *in vitro* study, fresh and ensiled Verko and Kometa rich in saponins alfalfa varieties mitigate methane production by reducing ruminal microbial populations, mostly protozoa and methanogens,
- different varieties and parts of the alfalfa (aerial, roots, or mixture) contain disparate individual saponins constituting the basis for the effectiveness of methane mitigation in *vitro*,
- replacing grass silage with alfalfa silage can inflect rumen microbial environment, mitigate methane production and enhance milk fatty acids composition, to some extent due to the saponin content in alfalfa silage in *in vitro* and *in vivo* experiments,
- more promising response would be expected with Kometa alfalfa silage, due to the higher saponin content.

3. Research objectives:

- to assess structural changes in alfalfa saponins during ensiling through analysis of saponins in both fresh and ensiled alfalfa, using ultrahigh performance liquid chromatography coupled with mass spectrometry,
- to examine if changes in saponin content were associated with changes in the basic nutrient composition of ensiled alfalfa,
- to investigate the effect of total saponins obtained from two alfalfa varieties, fresh and ensiled, on ruminal fermentation parameters, determined with the Hohenheim gas test,

- to explore the influence of individual saponins extracted from alfalfa on the methane production and total microbial populations, using *in vitro* batch culture system,
- to survey the impact of partial replacement of grass silage by Kometa or Verko alfalfa silage in a high-forage diet containing maize silage on ruminal fermentation parameters using RUSITEC system,
- to examine effect of alfalfa silage in the diet on *in vivo* ruminal fermentation, ruminal fatty acids composition, dry matter intake, and total-tract digestibility in experiments with rumen-cannulated cows,
- to survey the effect of saponins in alfalfa silage on milk production performance and milk fatty acid composition in dairy cows kept under commercial farm conditions.

4. Materials and methods

4.1. Plant material and ensiling process

Ten varieties of alfalfa (Verko, Kometa, Hunter River, Ever, Pomposa, Eugenia, Pieven, Radius, La Bella Campagnola and Sanditi) have been grown on plots of 100 m^2 for each variety in the experimental fields of the University of Life Sciences in Poznań for two years. Alfalfa was harvested at about 10% of the bloom stage in June and chopped into 1.5 cm particle length. Then, separately 50 kg of alfalfa were placed in a plastic container and mixed to consolidate the herbages. Samples were taken from the prepared material for chemical analysis. The remaining part of the alfalfa harvest was left in the field for 14 hours to wither. Then, about 2.6 kg of alfalfa were ensiled in separate, six microsilos (15 cm x 49 cm, 4 dm^3, plastic drums) which were sealed with inner and outer caps and stored for 8 weeks at 29 - 36 ° C temperature. Immediately before placing the materials into the silos, alfalfa was treated with the biological additive Agricol Sil (Microferm Ltd, UK). One gram (1 g) of the additive contained 10^{11} CFU of lactic acid bacteria: *Lactobacillus plantarum* DSMZ 16627, *Pediococcus acidilactici*

NCIMB 30005 and contained the enzyme producing strain *Lactobacillus paracasei* NCIMB 30151. The additive was prepared on the day of ensiling according to the manufacturer's recommendation, i.e., 0.01 g per 1 kg of fresh material (1 kg per ton), which had to be dissolved in the amount of 100 g per 20 l of water before use. At the end of the ensiling period, the silos were opened and samples were taken for further chemical analysis.

4. 2. Saponin extraction

The aerial and root parts of alfalfa (fresh and ensiled) were separately lyophilized using a Christ Gamma LSC freeze-drier (Martin Christ, Osterode am 220 Harz, Germany). Samples weight 100 mg were blended with diatomaceous earth (ASE Prep DE, Dionex, Sunnyvale, CA) and degreased with chloroform using a Soxhlet extractor (FOSS, Hillerød, Denmark). Subsequently, samples were extracted with 80% MeOH carried out with an accelerated solvent extraction system (ASE 200, Dionex, Sunnyvale, CA) under reduced pressure of 10 MPa and at 40 °C. The extracts, after filtering and evaporation to almost dryness, were purified using solid-phase extraction on a short glass column filled with the RP-C18 bed, previously dissolving them in 40% MeOH. Samples were eluted with 80% methanol and were evaporated as before under low pressure until the methanol was completely removed. The final product was lyophilized using a Christ Gamma LSC freeze-dryer and stored at -50 °C.

For quantitative analysis and determination of the saponins, the samples after extraction were dried to dryness under reduced pressure conditions, then dissolved in methanol and purified by solid phase extraction. The samples were then diluted with ten volumes of distilled water and added to Waters Sep-Pak Classic columns (360 mg, Waters, Milford, MA). The columns were washed with 5 mL of distilled water and then eluted with 5 mL of 80% MeOH. The eluate was evaporated to dryness, dissolved in pure methanol and stored at -20 °C.

4.3. *In vitro* experiments

4.3.1. Preparation of inoculum and incubation

For the Hohenheim gas test technique and the batch culture experiment, ruminal fluid was obtained from nine Polish Holstein – Friesian dairy cows, that were fed 20 kg of DM of a 600:400 forage: concentrate per day similar to *in vitro* experiment diet. Fresh ruminal fluid was strained through a four-layered cheesecloth into two Schott Duran® bottles (Schott North America, Elmsford, NY, USA) under anaerobic condition and at 39 °C temperature. Schott Duran® bottles were immediately transported to the laboratory, where they were placed in a water bath at 39 °C. Next the same volume rumen fluid from each cow (three cows in each treatment) were mixed and diluted with an artificial buffer solution (480 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4 \cdot 7H_2O$, 64 mg $CaCl_2 \cdot 2H_2O$, 50 mL of 8% Na_2CO_3, and 600 mg cysteine hydrochloride per liter buffer). The buffered rumen fluid was exposed to carbon dioxide gas for several minutes before being transferred to the experimental vessels.

For the rumen simulation technique experiment (RUSITEC) the fresh rumen inoculum was collected from the top, bottom, and middle parts of the rumen 3 hours after the morning feeding from three rumen-cannulated multiparous Polish Holstein-Friesian dairy cows in the fourth month of lactation. All cows were fed a TMR (at 24.4 kg of DM/d) similar to the control diet presented in **Publication 3, table 1.** Mixed rumen content (approximately 600 g) was transferred through four layers of cheesecloth, pour in a bottle purified with nitrogen gas, and instantly transported under anaerobic condition in 39 °C to the laboratory.

4.3.2. Hohenheim gas test

The Hohenheim gas test technique was conducted according to Menke and Steingass [30], with some modifications. Each time the experiment lasted 48 hours and was repeated three

times. Each group (control or experimental) consisted of five Hohenheim 100 mL glass syringes (Häberle LABORTECHNIK GmbH + Co., Lonsee-Ettlenschieß, DE). The division into control groups and experimental groups in the Hohenheim gas test experiment was presented in **Publication 2, table 2.**

4.3.3. Batch culture experiment

The batch culture experiment was conducted in the following manner. Briefly, 20 mL of inoculum was poured into heated incubation vessels already containing 0.2 g of substrate. The experiment was carried out in anaerobic conditions at pH 6.5 and 39 °C. The incubation flasks were closed with rubber stoppers and aluminum caps, placed in the incubator for 24 hours and mixed periodically. The preparation of eleven treatments were shown in **Publication 2, paragraph "Batch fermentation culture experiment".**

4.3.4. The rumen simulation technique (RUSITEC) experiment

The RUSITEC system consisted of three one-liter fermenters. Both experiments were performed in three incubation cycles. Each cycle consisted of five adaptation days and five sampling days. On the first day of each treatment, 720 mL of pooled strained ruminal fluid and 100 mL of prewarmed artificial saliva were poured into each fermenter. Then, 11 g DM of pooled ruminal solids and 11 g DM of experimental feed were added into perforated feed container inside of vessel in two individual nylon bags (70 × 140 mm; 100 µm pore size; Benetex, Poznań, Poland). In the next day, bags contained pooled ruminal solids was replaced by bags with feed substrate. Each time after 48 hours the bag with feed substrate was replaced with a new one, till the end of the run. After removing bag was rinsed with 50 mL artificial saliva to recover the microorganisms attached to the feed particles and artificial saliva was poured back to the fermenters. The entire fermenter was kept in a water bath at 39 °C, the constant stirring speed was 15 rpm using an impeller stirrer, and 99% purity carbon dioxide

gas was used to maintain anaerobic conditions. The artificial saliva was fed at a constant rate of 0.347 mL/min by an electronic peristaltic pump (Miniplus 3; Gilson, Middleton, WI, USA) to each fermenter. In the effluent vessels, the 10 mL of 10 M H_2SO_4 was poured to stop fermentation, after replacing feed bag the vessel has been subjected to nitrogen gas for 3 minutes at 3 L/min each time. The description of the individual experimental groups and controls was provided in **Publication 3, paragraph 2.1.1. *In vitro* experiments (Experiments 1 and 2).** All the forages used in the experiment were dried at 55 °C for one day, milled to less than 1 mm diameter to facilitate the rejection of fine particles lost through the pores of the nylon bag and totally mixed based on the dry matter basis amounts shown in **Publication 3, table 1.**

4.5. *In vivo* experiments

4.5.1. Rumen-cannulated dairy cows' experiment

An experiment using six multiparous lactating Polish Holstein-Friesian cows fitted with rumen cannulas (2 C, 4-inch, Bar Diamond, Parma, 239 Idaho, USA) was conducted in a replicated 3 × 3 Latin square design with three 26-day periods. The diets used in the experiment are presented in **Publication 3, table 1.** Cow were housed in individual tie-stalls, bedded with wood shavings, if necessary, with access to fresh water *ad libitum* and fed twice a day at 6:00 am and 6:00 pm. Cows were milked twice daily at 5:30 am and 5:30 pm. The sampling period was 22-26 days.

4.5.2. Commercial dairy cows' experiment

The experiment using fifty-four multiparous lactating Polish Holstein-Friesian dairy cows were prepared in a complete randomized design during 26-day period. The diets used in the experiment are presented in **Publication 3, table 1.** Cows were milked twice daily

at 5:30 am and 5:30 pm and fed at 6:00 am and 6:00 pm. Access to fresh water was *ad libitum*. The sampling period was the last 5 days of the experiment.

4.6. Sample analysis

4.6.1. Chemical analysis

Lyophilized samples of fresh and ensiled alfalfa and feed for further experiments were analyzed following AOAC methods: for dry matter (method no. 934.01), ash (method no. 942.05), crude protein (method no. 976.05, Kjel-Foss Automatic 16210 analyzer, Foss Electric, Hillerød, Denmark) and ether extract (method no. 973.18; Soxhlet System HT analyzer; Foss Electric, Hillerød, Denmark) [31]. Organic matter was considered as differences between dry matter and ash. Additionally, in the case of alfalfa samples, crude fiber was analyzed (method no. 978.10; Tecator Fibertec System I, Foss Electric, Hillerød, Denmark), and in the case of feed - ash free adjusted neutral detergent fiber was determined using heat-stable α-amylase and sodium sulfite (Fibertech 1020 Analyzer, Foss., Analytical AB, Höganäs, Sweden).

4.6.2. Saponin determination

Immediately prior to analysis, the samples were diluted 4 times with deionized and distilled water containing the internal standard and then centrifuged at 23 000 × g for 15 minutes. The analysis was performed on a Waters Aquity UPLC system equipped with a triple quadruple mass spectrometer (Waters TQD, Milford, MA, USA) and a Waters BEH C18 column (100 × 2.1 mm, 1.7 μm). The analysis of each sample was repeated three times, injecting one μl each time. The observed mass-to-charge ratios of saponins are shown in **Publication 1, table 1.** Analysis of the standards and plant extracts was performed on a Dionex Ultimate 3000RS chromatography system coupled to a Bruker Impact II HD (Bruker, Billerica, MA, USA) QTOF mass spectrometer. The spectral processing

was performed with Bruker Data Analysis 4.4 software. The calculated ionic formulas and the observed retention times were matched to the spectral library developed in-house as well as the Bruker Sumner MetaboBASE Plant Libraries version 1.0. A detailed description of the preparation of the analysis can be found in **Publication 1, paragraph "Saponin determination"**.

4.6.3. Rumen fluid sample analysis

The pH was measured using a pH meter (Elmetron CP-104, Zabrze, Poland) immediately after receiving the sample to the laboratory. Ammonia concentration was determined using the colorimetric Nessler method. The volatile fatty acids (VFA) were analyzed using a gas chromatograph (Varian CP 3380; Sugarland, TX, USA) with an injector at 120 °C, a flame ionization detector at 230 °C, and a capillary column (30 m × 0.25 mm; Agilent HP-Innowax, 19091N-133, Agilent Technologies, Santa Clara, CA, USA) mounted [26]. For identification of VFA peaks external standards (Fluka, Sigma Aldrich, MO, USA) were used. MS Work Station 5.0 was used for data processing. The digestible dry matter concentration was defined as the difference in dry weight of the feed substrate before and after incubation, after correction given residue in the blank control. Protozoa were counted under a light microscope (Primo Star no. 5, Zeiss, Jena, Germany) using a drop (10 µl or 100 µl) of buffered rumen fluid. Methanogens and all bacteria were quantified by fluorescence *in situ* hybridization (FISH). Three probes were used for their determination: SD-Arch-0915-aA-20 for all methanogens and two order-specific probes: SO Mmic-1200-aA-21 for *Methanobacteriales*, SF-Mbac 0310-aA-22 for *Methanobacteriales* and DAPI (40,6-diamidin-2-phenylindole) for total bacteria count. The Axio Imager M2 microscope (CarlZeiss Iberia, Madrid, Spain) was used for the visualization. A full description of the analysis is provided in **Publication 3, paragraph Ruminal microbial population.** For in *vitro* experiments, a gas chromatograph (SRI PeakSimple 310; Alltech, PA, USA)

containing a thermal conductivity detector and a Carboxen 1000 column (grid side 60/80, 15 FT × 1.8 INS.S; Supelco, Bellefonte, USA) was used [26]. In the experiment using cannulated dairy cows, the concentration of methane release was theoretically calculated as the fermentation balance for the measured molar fraction of volatile fatty acids and organic matter. In an *in vivo* experiment with production animals, methane emission was measured continuously with infrared methane analyzer (Servomex 4000 Series, Servomex, Jarvis Brook, UK) and employing 1210 Gfx modules using the gas filter correlation technique which is described in **Publication 3, paragraph Methane measurements**. Methane concentrations were recorded at 2 s intervals on a computer through the RS-232 port using software with a database system (AnaGaz, Wrocław, Poland). For measuring the fatty acid profile in feed and in the ruminal fluid the gas chromatograph (456-GC, Bruker, USA) equipped with a flame ionization detector and a 100-m fused-silica capillary column was used (0.25 mm id; coated with 0.25 μm Agilent HP; Chrompack CP7420; Agilent Technologies, Santa Clara, CA, USA) [26]. The peaks were identified by comparison with the retention times of the corresponding FAME standards (37 FAME Mix, Sigma Aldrich, PA, USA) using Galaxie Work Station 10.1 (Varian, CA, USA). Fatty acid compositions were expressed as g / 100 g total FA.

4.6.4. Milk analysis

Daily milk yields were recorded while sampling using a milk meter (WB Ezi-Test Meter 33 kg; True-Test, Manukau, New Zealand). Milk basic parameters were immediately analyzed by infrared analysis (MilkoScan 255 A/S N, FossElectric, Hillerød, Denmark). The fatty acid profile was analyzed in the same way as for the feed and rumen fluid trials.

4.7. Statistical analysis

In **Publication 1**, IBM SPSS Statistics 22 was used for analysis and Duncan's post-hoc test was used based on one-way ANOVA. A completely randomized design with six

replications per treatment was used. Differences were considered significant at $P \leq 0.05$. The PROC CORR SAS procedure was used to correlate the saponin content and the chemical composition of fresh alfalfa and silage. One-way correlations were found for $P < 0.10$. The stepwise PROC REG option was then used to identify the variables in this list that contributed significantly to the parameter prediction. The results for the individual alfalfa silages were compared by calculating the Pearson correlation coefficients between the concentration of saponins and the chemical composition of each of the alfalfa samples. Statistical analysis of data of Hohenheim gas test were based on a randomized complete block design proceed on the model shown in **Publication 2, paragraph "statistical analysis"**. Next, data were examined using a two-way ANOVA with Tukey's range test. In the case of batch culture experiment all data were analyzed using a one-way ANOVA. In the last *in vitro* experiments, the data from the RUSITEC were examined using a model which contained treatment fix effect, and two random effects: an incubation run effect (block) and a fermenter effect. In *in vivo* experiment using cannulated dairy cow data from intake, digestibility, and ruminal fatty acid composition were analyzed with the model which as fixed effects incorporated treatment and period and square and cow within square as the random effects. The ruminal fermentation characteristic data were examined as reproducible measures using the model above, with except, that sampling time (i.e., hour) and treatment × sampling time were fixed effects. Data from an experiment using commercial dairy cows were analyzed using a model that considered the fixed effect of treatment and the random effect of the cow within the treatment. In Hohenheim gas test and both *in vivo* experiments, the sums of squares for treatment effects were further separated into single degree of freedom comparisons to test the significance of preplanned contrasts: the effect of the experimental diet in comparison to the controls and the experimental diets among themselves. All *in vitro* analyses were performed using SAS software (version 9.3; SAS Institute Inc., Cary, NC, USA) and *in vivo*

analyses were carried out using PROC MIXED of SAS (SAS 9.2; SAS Institute, Cary, NC, USA). Values significantly different ($P < 0.05$) were labeled in tables within rows with different lowercase letters.

5. Results

The concentration of OM, ash, CP, CBF and CF differed slightly between the fresh varieties (**Publication 1, table 2**). The concentration of DM, OM, and CF increased with the exception of the Sanditi variety in the ensiled material, while the ash, CP, and CFR remained at the same level as in the fresh material (**Publication 1, table 3**). These data show that most of the chemical parameters of both types of plants materials are similar to those presented in other publications. This shows that the ensiling process has proceeded in a standard way. There were significant differences in the CP content between the cultivars in the fresh material ($P <0.033$), which was not observed in the case of the ensiled material. A comparison of DM in fresh and ensiled alfalfa cultivars shows that the concentration was on average 25% higher in the ensiled material, except for Pieven and Sanditi cultivars, where the increase in DM was about 10% compared to fresh material. Pearson's correlation coefficients between the saponin content (mg / g DM) and the chemical composition of alfalfa silage and fresh material shows strong correlation was observed between the CP content and the saponin content in fresh alfalfa varieties (**Publication 1, table 4)**. Ensiling alfalfa resulted in a significant increase in the measurement of total saponin content in each ensiled alfalfa variety compared to fresh material (**Publication 1, figure 2**). The lower the initial concentration of saponins in the fresh material of alfalfa variety, the higher the concentration of total saponins in their ensiled version. In this study, we also observed changes in individual saponins during the ensiling process into alfalfa (Publication 1, figure 3). Three of the zanhic acid glycosides (ZATA, ZATB, ZAU) were almost completely degraded during this process. On the other hand, the other two, ZAGA and ZAG, significantly increased their concentration by ensiling.

Of the seven medicagenic acid glycosides tested, two of them (MAGA and MAG1) practically ceased to appear in the samples of ensiled material, while the remaining medicagenic acid glycosides remained practically unchanged. In the case of glycosides of soyasapogenol B, their behavior was different. The glycoside SSI content decreased as the glycoside SSII content increased in the ensiled alfalfa material. Next of the purposes was to investigate the effect of total saponins obtained from two disparate alfalfa varieties, fresh and ensiled, on rumen fermentation parameters. A study using the Hohenheim gas test proved the limiting effect of saponins on microbial population and ruminal fluid parameters. Experiment with fresh Kometa and Verko alfalfa shown a linear reduction of total protozoa and methanogens count (**Publication 2, table 2 and 4**). More promising results, decrease in protozoa levels and in all methanogen counts, were obtained by adding the ensiled Verko and Kometa alfalfa (**Publication 2, table 4 and 6**). In the case of bacteria, there has been an increase in their population. However, the ensiled Kometa alfalfa had the strongest effect on methane production after 24 hours and 48 hours after incubation and total gas emission (**Publication 2, table 6**). For this reason, Kometa alfalfa was used for batch culture experiment. Almost every individual saponin has been shown to have methane production limiting properties (**Publication 2, table 8**). The best results were obtained in samples of extracted saponins from the roots: 3- Glc,28- Glc Ma, medicagenic, 3-Glu Ma. All root saponins and two out of four alfalfa leaf saponins had statistically significant antiprotozoal, antibacterial and antimethanogenic effects (**Publication 2, table 9 and 10**). Replacing grass silage with alfalfa silage in a high-forage diet containing maize silage did not significantly affect total gas production and ruminal microbial count. The assumption was confirmed for the decreases in the pH level and methane concentration and for the increases in digestible dry matter with Kometa alfalfa silage treatment (**Publication 3, table 3**). However, it led to an increase in ammonia and changed the ratio of acetate:propionate (A:P) and individual volatile fatty acids in both experiments in RUSITEC

fermenters (**Publication 3, table 2 and 3**). The addition of alfalfa silage in the diets of the rumen- cannulated dairy cows influenced positively the ruminal fermentation characteristic, as expected. Both alfalfa varieties decreased the total bacteria, total protozoa (including *Holotricha* and *Entodiniomorpha*), and the total methanogens. Moreover, there was a decrease in methane production and the total VFA concentration, but there was an increase in ammonia in both experimental groups (**Publication 3, table 4**). Alfalfa silage increases the concentration of unsaturated fatty acids such as C12:0, C14:0, and C16:0 in ruminal fluid (**Publication 3, table 5**). Despite this, Kometa alfalfa silage decreased the concentration of total saturated fatty acids in the rumen and increased monounsaturated and polyunsaturated fatty acids. However, the concentration of C18:0 decreased as the effect of experimental diets. Increase in ruminal C14:1, cis-9, cis-12 C18:2 and trans-10, cis-12 C18:2 was shown in alfalfa silage replaced diet. The higher response was observed when Kometa alfalfa silage was used. The response to Kometa alfalfa silage diet was greater than the response to Verko alfalfa silage diet in milk production and methane production in the lactating dairy cows (**Publication 3, table 6**). The increase in milk protein yield, milk lactose content, and the decrease in methane emissions caused by the action of alfalfa silage have been proven. The results show that alfalfa silage diet led to increased concentration of C12:0, C14:0, and C16:0, but decreased C18:0 in milk (**Publication 3, table 7**). Replacing grass silage with alfalfa silage increased C14:1 and C16:1. The milk fat content of cis-9 C118:1, trans-10 C18:1, trans-11 C18:1, cis-9, cis-12 C18:2 and trans-10, cis-12 C18:2 decreased when diet included alfalfa silage. The experimental diets led to decreased milk fat concentrations of total monounsaturated fatty acids and polyunsaturated fatty acids, when the total saturated fatty acids concentration increased in the milk, where the responses were lower in Kometa alfalfa silage.

6. Discussion

The correlation between the saponin content and the chemical composition of ensiled and fresh alfalfa material confirmed that the ensiling process affects the final saponin content and protein concentration in the plant material (**Publication 1, table 3**). An increase in saponins has also been demonstrated in each of the ten alfalfa cultivars (**Publication 1, figure 2**). Our results differ from the previously published studies on alfalfa silage, where a decrease in the concentration of saponins in the ensiled plant material was recorded [32]. The authors of cited publication concluded that enzymatic hydrolysis occurs during the ensiling process, but they were unable to detect medicagenic acid or zahnic acid aglycones. They suggested that the changes in saponins during the ensiling could be better explained with the advancement of technology in chemical analysis. There are two possible explanations for such different results. The first may be the loss of biomass in alfalfa during ensiling, usually in the form of gases and effluents [33]. In this study, there were no waste effluents, and the dry matter increased in all alfalfa varieties due to the ensiling process. Another explanation could be the better availability of chemicals in ensiled alfalfa. Saponins show a strong affinity to plant, animal, and fungal cell membranes, forming complexes with sterols [34]. The enzymatic reactions during the ensiling process can break these bonds and make the saponins more accessible. Earlier studies have shown that the most important of saponin from the point of view of animal nutrition and the most biologically active saponin of alfalfa are glycosides of medicagenic acid [18]. Therefore, it was important to identify and measure the concentration of these substances in ensiled varieties of alfalfa (**Publication 1, figure 3**). Our results show that most glycosides of medicagenic acid have undergone chemical transformation (hydrolyzed or oxidized) in various ways in the ensiling process. The same was the case with glycosides of zanhic acid, where most of them were completely hydrolyzed during ensiling. The exceptions are ZAGA and ZAG, which significantly increased their

concentration in ensiled alfalfa. It is suspected that carbon C18 in two glycosides of medicagenic acid (MAGA and MAG1) is oxidized during the ensiling process and the formation of ZAGA and ZAG, respectively. Changes in two soyasapogenol B derivatives were also observed during the ensiling of alfalfa. Namely, the concentration of SSI decreases during this process, while the concentration of SSII increases. It is suspected that SSI converts to SSII during ensiling, because these compounds differ only in one carbohydrate residue. The effect on the basic ruminal parameters of individual saponins from ensiled alfalfa was investigated in later publications. According to the results of the experiment (**Publication 2, table 2-6**) using the Hohenheim gas test, the ensiled Kometa alfalfa, was more effective in reducing methane production and reducing the protozoa and methanogens populations. These can be explained by the increased concentration of saponins in the ensiled form of alfalfa (**Publication 2, table 1**). The antiprotozoal activity of saponins affects the production of methane in two ways. By limiting the population of protozoa, it reduces the amount of hydrogen in the rumen needed for the production of methane by methanogens [35]. Additionally, there are methanogens that form a symbiotic relationship with the protozoa (protozoa-associated methanogens), that convert malate to hydrogen. About 15% of protozoa contain methanogens in their cells, so the destruction of their cell membranes by saponins by causing cell lysis would also reduce the population of methanogens, and thus the production of methane [36, 37]. The increased bacterial population could be due to the reduced number of protozoa that are natural predation to bacteria [38]. Another explanation could be the fact that some of saponins could stimulate growth of amount of cellulolytic bacteria population [39]. However, later studies exclude this theory because of the smaller bacterial content in experimental groups *in vitro* and *in vivo* experiments. A study using individual saponins as experimental trials showed that the reduction in total protozoa, bacteria, total methanogens, *Entodiniomorpha*, *Methanobacteriales*, and *Methanomicrobiales* counts (**Publication 2,**

table 9 and 10) was accompanied by decreased methane production. The results proved that saponins extracted from the alfalfa root had the strongest antiprotozoal effect, and thus antimethanogenic and limiting the production of methane. The different responses of individual saponins may have been due to a different structure. Saponins consist of a hydrophobic aglycone backbone linked to hydrophilic sugar chains [40]. Another sugar moiety may lead to a more or less potent saponin effect [6]. For example, monodesmoside saponins are generally more active than bidesmoside saponins [41]. Steroid saponins have a better effect in limiting methane production than triterpenoid saponins [38]. Summarizing, the presence of various substituents in saponins, as well as differences in the number of bonds and the amount of sugar chains are responsible for the different bioactivity of these substances [42]. The increase in digestible dry matter may have been caused by an increase in the crude protein content in the diet with the addition of alfalfa silage, thereby causing an increase in the total degraded dry matter fraction (**Publication 3, table 2**) [11]. A lower A:P ratio may be associated with a lower neutral detergent fiber (NDF) content [43]. The increase in ammonia in the experimental groups resulted in a decrease in pH, and then inhibition of the use of ammonia by the rumen microorganisms (**Publication 3, table 2-3**) [44]. In a study using RUSITEC fermenters, a significantly lower population of rumen microorganisms was observed than in previous *in vitro* and *in vivo* studies. One of the reasons for this may be the exposure of the fermenter to atmospheric oxygen and the process of scrubbing free-living microorganisms [45]. The increase in propionate and butyrate in alfalfa groups could be due to a shift of hydrogen from the methane production pathway and is, therefore, available for propane production [46]. In addition, the lowered pH created favorable conditions for propanoate-producing bacteria that inhibited populations of other microbes, thereby reducing the A: P ratio as confirmed by studies on rumen-cannulated dairy cows (**Publication 3, table 4**) [47]. In **the publication 3,** the research confirmed, as in the case

of *in vitro* studies, that the replacement of grass silage by alfalfa silage in the dairy cows' diet through the limiting effect of saponins on the population of protozoa-associated methanogens, which are responsible for as much as 37% rumen methane, reduces methane production [48]. Feeding a diet containing Verko alfalfa silage led to a decrease in fat content and fat yield, which may have been due to the decreased NDF content of this diet, thereby reducing chewing activity, resulting in lowering the pH, which may result in increasing the risk of milk fat depression (**Publication 3, table 6**) [49]. However, no such results were observed in the diet replacing grass silage with Kometa alfalfa silage. The limiting effect of alfalfa silage in diet of commercial dairy cows on methane emission was also found, where a stronger response was noted with Kometa alfalfa silage. As expected, the experimental groups had a positive effect on the amount of cis-9 cis-12 C18:2 and trans-10 cis-12 C18:2 acids in the ruminal fluid (**Publication 3, table 5**). A more promising fatty acid composition in milk was found in groups containing Kometa alfalfa silage as an additive to dairy cows' diets (**Publication 3, table 7**). It should be noted that the fatty acid profile in the rumen does not reflect that of the fatty acids in milk, that the composition of acids is not identical to that of the duodenum, which is the main site for fatty acid absorption into the blood [26]. In addition, the process of biohydrogenation of vaccenic acid to C18:0 limited the formation of polyunsaturated fatty acids in ruminant products, such as milk [50]. The modification in the fatty acid profile is also influenced by changes in the microorganism's population directly involved in the biohydrogenation process [26]. Attempts to improve the fatty acid profile in milk therefore require understanding the relationship between the supply of dietary lipids, rumen fermentation and metabolic changes in the blood, liver, and finally in the mammary glands [51].

7. Conclusion

The ensiling alfalfa process increases the content of bioactive compounds - saponins and changes the composition of individual saponins in the plant material. It has been shown that saponins, due to their antiprotozoal, antimethanogenic andantibacterial properties, modulate the processes taking place in the ruminal fluid, thus changing the volatile fatty acid composition, the profile of fatty acids, and limiting the production of methane. The inclusion in the diet the alfalfa silage containing saponins also affects the milk fatty acid profile. The above statements are confirmed by studies conducted either *in vitro* or *in vivo*. It has been shown that ensiled Kometa alfalfa has the strongest potential to mitigate methane emission, both *in vitro* and *in vivo* in experiments with highly-productive dairy cows, without negatively affecting ruminal fermentation. Additionally, ensiled Kometa alfalfa had a more favorable effect on the milk fatty acid profile. In summary, the Kometa alfalfa silage as a source of saponins can be successfully used as a valuable component of the dairy cows' diet that additionally reduce rumen methane emission.

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Structural and quantitative changes of saponins in fresh alfalfa compared to alfalfa silage

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Abstract

BACKGROUND: Alfalfa (*Medicago sativa*) is an important legume used in animal feed. The primary objective of the present study was to analyze and trace the individual saponins in fresh and ensiled alfalfa of ten varieties, with the aim of evaluating the structural changes that saponins undergo during ensiling. A secondary objective was to examine whether any of the changes in saponin content were associated with changes in the basic nutrient composition of the ensiled alfalfa.

RESULTS: The total saponin concentration increased when the fresh alfalfa was processed into silage. Three of the zanhic acid glycosides were degraded substantially, whereas the other two increased in all the tested varieties. Five of the seven medicagenic acid glycosides were not changed, whereas the remaining saponins completely disappeared. Finally, two glycosides of soyasapogenol B displayed an inverse relationship to each other.

CONCLUSION: The nutrient content of fresh material and silages remained relatively constant, with some variations in the crude protein content of the selected alfalfa varieties. The total concentration of saponins increased two- to three-fold after ensilation over the levels present in fresh alfalfa material. The increases in saponin concentration showed a negative linear relationship between protein on a dry matter basis and saponin content. These findings are significant because no known published data have shown this transformation in the ensiling of alfalfa.

Keywords: alfalfa; silage; saponin; ensilation; liquid chromatography; glycosides

INTRODUCTION

Medicago sativa, commonly known as alfalfa and lucerne, is one of the most valuable legume plants that can be widely grown in a temperate climate. As a result of its high protein content, it provides excellent pasture for ruminants and, accordingly, it is utilized in the form of green alfalfa feed (AF) for grazing or as hay or pellets. However, in moist climates where field drying is challenging and risky, farmers instead process it into alfalfa silage (AS).[1] This process is rather complex in the case of alfalfa because of the low carbohydrate contents of the plant, as well as its high buffering capacity, which makes ensiling resistant to a drop in pH.[2] Furthermore, a large proportion of the crude protein in alfalfa can be degraded to soluble non-protein nitrogen (N) in the course of ensiling.[3] In case of monogastric animals, there is some evidence that their performance is rather weak when fed with alfalfa pasture.

Alfalfa saponins (SA) are pentacyclic triterpenes. These compounds occur as glycosides of several aglycones, including medicagenic acid (MA), zanhic acid (ZA), hederagenin (H), and soyasapogenol (SS).[4,5] Saponins can occur in a monodesmosidic form (with one sugar chain attached to the aglycone), a bisdesmosidic form (with two attached sugar chains) or even a tridesmosidic form (with three sugar chains attached). Saponins present differential biological activity, depending on the aglycone structure and the length and composition of the carbohydrate side-chains. The activities that are most important from the nutritional point of view are foaming properties, hemolytic and antimicrobial properties, throat-irritating effects, and modulatory effects on the permeability of the intestinal membrane. The hemolytic and antimicrobial activities of SA strongly depend on the aglycone and sugar chain structures.[6] The most active of all AS are the glycosides of MA, and the monodesmosides show higher activity than bisdesmosides. ZA glycosides show mild activity in this respect, whereas the glycosides of SS are inactive. Throat irritation has been shown to be the main feature of ZA glycosides,[5] and this may help explain why some animals avoid saponin-containing sources of pasture. Some studies have

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reported that alfalfa saponins are biologically active and may inhibit microbial fermentation and rumen synthesis.[7] However, to understand the interactions between saponins and microbial populations, knowledge of the relationship between the chemical structure and activity of the saponins is required. This would allow the fermentation processes in the rumen to proceed properly.[8]

Additionally, given the broad diversity of structures and activities of saponins, the plant materials used for animal feed should be evaluated for their saponin contents. In particular, feeds produced by compacting plant material should be carefully investigated because, in such cases, significant enrichment of the feed with saponins and other antinutritional components may occur. On this basis, we hypothesize that there is a higher saponin content in ensiled alfalfa than in fresh material as a result of the processes occurring during ensiling. The present study therefore focuses on analyzing SA in both AF and AS, using ultra high-performance liquid chromatography combined with mass spectrometry (MS) to evaluate structural changes in SA during ensiling. In addition, because of the potentially high nutritional value of both fresh and ensiled alfalfa, a secondary objective was to examine whether any changes in saponin content were associated with changes in the basic nutrient composition in ensiled alfalfa.

MATERIALS AND METHODS

Plant material

Seeds of ten varieties of alfalfa Verko (German), Kometa (Polish), Hunter River (Australia), Ever (unknown origin), Pomposa (Italian), Eugenia (Italian), Pieven (unknown origin), Radius (Polish), La Bella Campagnola (Italian) and Sanditi (Dutch) were purchased from a local market in Poland. The plants were grown in plots of 100 m^2 for each variety in experimental fields at Poznań University of Life Sciences in 2014. Plants were harvested during the second year of cultivation at the first cut in June. Alfalfa was harvested at about the 10% bloom stage and chopped to a particle length of 1.5 cm. Approximately 50 kg of each variety from a different part of the experimental plot was collected separately, placed in a plastic container and mixed to consolidate the herbages. Six independent samples of fresh alfalfa were taken from the prepared material for further chemical analysis. The remaining harvested alfalfa was wilted for 14 h on the plot. After wilting, the alfalfa was ensiled in six microsilos (plastic drums of 4 dm^3 each, diameter 15 cm, height 49 cm) treated with the biological additive Agricol Sil. One gram of the additive contained 10^{11} colony-forming units of lactic acid bacteria namely, *Lactobacillus plantarum* DSMZ 16627 and *Pediococcus acidilactici* NCIMB 30005, as well as an enzyme-producing strain, *Lactobacillus paracasei* NCIMB 3015. The additive was prepared on the day of ensiling, applied in solution (i.e. diluted with water, 100 g per 20 L) at 0.01 g per 1 kg (1 kg per 100 tons) of fresh weight. Before being packed into the silos, the additive was spread on the chopped forage in 50-kg plastic containers and thoroughly mixed. Approximately 2.6 kg of chopped fresh alfalfa was immediately packed into plastic laboratory silos, sealed with two screw tops (internal and external) and stored at ambient temperature (29–36 °C) for 8 weeks. At the end of the ensiling period, the silos were opened and samples were taken for chemical analysis. In total, 120 samples were collected: 60 fresh alfalfa and 60 ensiled. Six replicates of each variety were used for further chemical analysis. Ammonia, pH, volatile fatty acids, and lactic acid were analyzed in the alfalfa silage, in accordance with AOAC methods.[9] Samples of the silage were collected and lyophilized for saponin analysis from each silo, for each variety.

Chemical analysis

Lyophilized samples of fresh and ensilaged alfalfa were analyzed in accordance with AOAC methods[9] for dry matter (DM) (method no. 934.01) and ash (method no. 942.05). Crude protein (CP) was determined with a Kjel-Foss Automatic 16210 analyzer (Foss Electric, Hillerød, Denmark) (method no. 976.05); ether extract (EE) with a Soxhlet System HT analyzer (FOSS, Hillerød, Denmark) (method no. 973.18); and crude fiber (CF) using a Tecator Fibertec System I (FOSS, Hillerød, Denmark) (method no. 978.10). Organic matter (OM) was calculated from the difference between DM and ash.

Saponin extraction

Quantitative analysis of the saponins was based on previous protocols.[10,11] Briefly, 100 mg (dry weight) samples of lyophilized plant material were mixed with diatomaceous earth (ASE Prep DE, Dionex, Sunnyvale, CA, USA) and extracted with 80% MeOH in the stainless steel extraction cells of an accelerated solvent extraction system (ASE 200; Dionex). Extraction was carried out at an operating pressure of 10 MPa and at 40 °C. After evaporation to dryness under reduced pressure, samples were dissolved in methanol and purified using solid phase extraction. The samples were then diluted with ten volumes of distilled water and added to Sep-Pak Classic columns (360 mg; Waters, Milford, MA, USA). The columns were washed with distilled water (5 mL) and subsequently eluted with 80% MeOH (5 mL). The eluate was evaporated to dryness, dissolved in pure methanol and stored at −20 °C.

Saponin determination

Immediately prior to analysis, the samples were diluted four times with distilled, deionized water containing an internal standard (digoxin, final concentration: 2 ng μL^{-1}) and centrifuged at 23 000 × *g* for 15 min. Analysis was performed on a Aquity UPLC system (Waters) equipped with a triple quadruple mass spectrometer (TQD; Waters). The mobile phase was composed of solvent A (0.1% formic acid in distilled and deionized water) and solvent B (liquid chromatography-MS grade acetonitrile containing 0.1% formic acid). A binary gradient pump (Waters) delivered the mobile phase at 0.3 mL min^{-1} onto a BEH C18 column (Waters) (100 × 2.1 mm, 1.7 µm) held at 50 °C. Initial mobile phase conditions for the gradient were 75% of solvent A and 25% of solvent B. One minute after the injection, the concentration of solvent B was ramped concavely to 55% over 23 min. Finally, the column was washed for 2 min with 100% of phase B and re-equilibrated with 25% phase B for 4 min.

One microliter was injected from each sample and the analysis of each sample was repeated three times. The column's effluent was introduced into the electrospray ion source of the mass spectrometer, operating in negative ion mode. The ion source parameters were: capillary voltage at 3.1 kV, extractor at 3 V, RF lens at 100 mV, source temperature of 140 °C, desolvation temperature of 350 °C, desolvation gas flow of 700 L h^{-1} and cone gas flow of 50 L h^{-1}. The collision cell entrance was set to −2 and the exit to 0.5 arbitrary units. The collision energy used was 5 eV, to minimize fragmentation of deprotonated ions and achieve sufficient sensitivity in the single ion monitoring mode. The observed mass-to-charge (*m/z*) ratios of the saponins are shown in Table 1. The final dwell time was 0.02 s, allowing for 10–25 data points per average chromatographic peak. Data acquisition and processing were performed using MassLynx 4.1 SCN 849 software (Waters).

Table 1. Chromatographic characteristics of analyzed saponins

No.	RT(min)	m/z[M-H]$^-$	Cone voltage (V)	Compound ID	Calibrationstandard
ZATA	4.2	1545	130	3-GlcGlcGlc,28-AraRhaApiXyl zanhic	ZATA
ZATB	4.6	1545	130	3-GlcGlcGlc,23-Ara,28-AraRhaXyl zanhic acid	ZATB
ZAGA	5.6	1103	105	3-GlcA,28-AraRhaXyl zanhic acid	MAG1
ZAU	6.1	1221	120	3-Glc,28-AraRhaAraXyl zanhic acid	MAG1
ZAG	6.7	1089	100	3-Glc,28-AraRhaAraXyl zanhic acid	ZATA
IS	7.6	779	65	digoxin (internal standard)	
MAA	8.9	1529	120	3-GlcGlcGlc, 28-AraRhaApiXyl medicagenic acid	ZATA
MAB	9.3	1529	120	3-GlcGlcGlc, 28-AraRhaAraRha medicagenic acid	ZATB
MAC	10.6	1219	120	3-GlcA, 28-AraRhaApiXyl medicagenic acid	ZATA
MAGA	11.1	1087	100	3-GlcA,28-AraRhaXyl medicagenic acid	MAGA
MAG2	11.2	1235	120	3-GlcGlc,28-AraRhaXyl medicagenic acid	MAG1
MAG1	11.9	1073	100	3-Glc,28-AraRhaXyl medicagenic acid	MAG1
MAMG	15.7	911	92	28-AraRhaXyl medicagenic acid	MAG1
SSI	18.3	911	92	3-GlcAXylRha soyasapogenol B	SSII
SSII	18.4	941	92	3-GlcAGalRha soyasapogenol B	SSII

Saponins were identified based on their high-resolution MS and MS/MS spectra. Analysis of the standards and plant extracts was carried out on an Ultimate 3000RS chromatographic system (Dionex) coupled with an Impact II HD (Bruker, Billerica, MA, USA) quadrupole time of flight mass spectrometer. Chromatographic conditions were as described above. The mass spectrometer operated in negative ion electrospray mode. Linear (centroid) spectra were acquired over a mass range from m/z 150 to m/z 2000. The mass spectrometer parameters were: a capillary voltage of 3 kV; dry gas flow at 6 L min^{-1}; dry gas temperature of 200 °C; nebulizer pressure of 0.7 bar; collision RF at 600 V; transfer time of 70 µs; and prepulse storage of 7.0 µs. The collision energy was set automatically from 20 to 120 eV, depending on the m/z of the fragmented ion. The data were calibrated internally with a 5 mmol L^{-1} sodium formate solution (in 50% 2-propanol) introduced to the ion source via a 20-µL loop at the beginning of each separation. Spectral processing was performed using DataAnalysis, version 4.4 (Bruker). The calculated ion formulas and observed retention times were matched against a spectral library developed in-house and Sumner MetaboBASE Plant Libraries, version 1.0 (Bruker) (Table 1).

Statistical analysis

SPSS, version 22 (IBM Corp., Armink, NY, USA) was used for analysis and Duncan's post-hoc test was employed based on one-way analysis of variance. $P < 0.05$ was considered statistically significant. A completely randomized design with six replicates per treatment was employed. The saponin content in plants was considered appropriate for studying factors associated with nutrient contents in the fresh and ensiled alfalfa. The PROC CORR procedure of SAS (SAS Institute Inc., Cary, NC, USA) was used to correlate the saponin content and chemical composition of alfalfa fresh and silage, including organic matter, ash, protein, fiber, fat, pH, ammonia, lactic acid, acetic acid and butyric acid contents. Single-factor correlations were reported for $P < 0.10$. The stepwise option of PROC REG was then used to identify the variables from this list that significantly contributed to predicting the parameters. The results for individual alfalfa silages were compared by calculating Pearson's correlation coefficients between the saponin concentration and the chemical composition of the fresh and ensiled alfalfa of each variety.

RESULTS

Chemical composition of raw and ensilaged alfalfa

The chemical composition of the AF fresh material is presented in Table 2. The concentration of DM in the varieties ranged from 242 to 278 g. The concentration of OM, ash, CP, EE, and CF differed slightly between varieties, although these differences were generally not high. The chemical composition of the alfalfa silages is given in Table 3. The concentration of DM, OM, ash, and EE increased, while CP, and CF remained at the same level as in the fresh material.

Overall, the nutrient content of the fresh material and silages was relatively constant; however, the CP content of AF was more variable ($P < 0.033$) than that for AS. The protein content of the individual varieties showed similar tendencies in AF and AS. For unexpected reasons, the level of ammonia-N in AS increased by up to 10.3% of the total N in the Sanditi variety. These data show that most of the chemical parameters of AF and AS are similar to those reported previously, indicating that the ensiling process was a standard one. There were significant differences in CP content between varieties in AF, whereas such differences were not significant in AS. The varieties also differed (not significantly in the case of DM content). Comparing DM in AF and AS indicates that, on average, this concentration was 25% higher in AS, except for the Pieven and Sanditi varieties, for which this increase was only 10%.

Pearson correlation coefficients between saponin content (mg g^{-1} DM) and the chemical composition of the alfalfa silages and fresh material are presented in Table 4. There was a strong correlation between CP and saponin content in the AF. The regression was described as protein (g kg^{-1} DM) = 239.9 – 4.82 × saponin (mg g^{-1} DM; $r^2 = 0.613$; $P = 0.007$) (Fig. 1).

Total saponin concentration

The results for total saponins ranged from 0.7 mg g^{-1} DM in Eugenia to 3.3 mg g^{-1} DM in Kometa and Hunter River varieties. The total saponin concentration (TS) in fresh alfalfa samples and in the equivalent ensilaged samples is presented in Fig. 2. The TS increased in AS over its value in the AF. This increase depended on the variety, ranging from 1.7 times for Hunter River to 4.4-fold for Eugenia.

Ensiling alfalfa resulted in a substantial increase in the measured saponin content in all ten varieties. The saponin concentration in

Table 2. Chemical composition of alfalfa fresh material

Items	Verko	Kometa	Hunter River	Ever	Pomposa	Eugenia	Pieven	Radius	La Bella Campagnola	Sanditi	SEM	*P*-value
DM (g kg^{-1})	242	268	242	237	266	252	279	244	272	256	6.723	0.929
OM (g kg^{-1} DM)	807	808	801	802	804	803	800	799	812	828	3.613	0.879
Ash (g kg^{-1} DM)	118	119	117	116	115	119	115	114	110	112	1.06	0.601
CP (g kg^{-1} DM)	210 abc	220 abc	202 bc	198 c	211 abc	224 ab	230 a	226 a	208 abc	203 bc	2.631	0.033
CF (g kg^{-1} DM)	265	253	276	288	281	269	276	278	290	295	4.094	0.513
EE (g kg^{-1} DM)	14.9	14.1	14.2	12.9	13.4	11.7	13.6	12.9	12.8	11.9	0.264	0.117

DM, dry matter; OM, organic matter; CP, crude protein; CF, crude fiber; EE, ether extract.
Values within rows with different lowercase letters are significantly different ($P < 0.05$).
SPSS, version 22 (IBM Corp.) was used for analysis and Duncan's post-hoc test was used based on the one-way analysis of variance.

Table 3. Chemical composition of alfalfa silages

Items	Verko	Kometa	Hunter River	Ever	Pomposa	Eugenia	Pieven	Radius	La Bella Camp	Sanditi	SEM	*P*-value
DM (g kg^{-1})	346	336	350	336	364	358	307	325	367	294	17.297	0.998
OM (g kg^{-1} DM)	867	874	865	868	863	870	872	872	878	876	2.193	0.926
Ash (g kg^{-1} DM)	108	123	126	124	126	122	121	119	115	129	2.834	0.93
CP (g kg^{-1} DM)	211	213	207	212	211	226	228	216	215	213	2.088	0.515
CF (g kg^{-1} DM)	259	254	265	273	271	264	263	273	282	275	6.092	0.998
EE (g kg^{-1} DM)	21.2	19.8	21	18.8	19.1	17.9	21.1	20.8	19.6	23.1	0.759	0.963
pH	4.56	4.59	4.5	4.6	4.61	4.44	4.82	4.73	4.63	4.76	0.047	0.841
Ammonia-N (% of total N)	5.95 bc	5.34 bc	6.23 bc	5.72 bc	4.77 c	7.37 b	7.16 bc	6.32 bc	5.48 bc	10.3 a	0.335	0.002
Lactic acid (g kg^{-1} DM)	28.1	30.2	23.7	31.9	51.3	41.7	61.6	44.5	36.2	57.6	2.202	0.173
Acetic acid (g kg^{-1} DM)	25.1	21.8	22.3	27.1	18.5	32.3	32.5	32.6	29.1	49.1	4.512	0.404
Butyric acid (g kg^{-1} DM)	2.90	7.58	7.90	8.52	8.77	8.29	13.8	13.7	12.5	7.42	2.001	0.873

DM, dry matter; OM, organic matter; CP, crude protein; CF, crude fiber; EE, ether extract.
Values within rows with different lowercase letters are significantly different ($P < 0.05$).
SPSS, version 22 (IBM Corp.) was used for analysis and Duncan's post-hoc test was used based on the one-way analysis of variance.

Table 4. Pearson correlation coefficients between saponin content (mg g^{-1} DM) and chemical composition of fresh material and alfalfa silages

Parameters	Fresh		Silage	
	r	*P*	*r*	*P*
Organic matter (g kg^{-1})	−0.199	0.582	−0.269	0.452
Ash (g kg^{-1} DM)	0.431	0.214	0.437	0.206
Protein (g kg^{-1} DM)	−0.4	0.252	−0.783	0.007
Fiber (g kg^{-1} DM)	−0.413	0.235	−0.181	0.617
Fat (g kg^{-1} DM)	0.817	0.004	−0.042	0.908
pH	–	–	−0.139	0.702
Ammonia (mmol L^{-1})	–	–	−0.629	0.051
Lactic acid (mmol L^{-1})	–	–	−0.503	0.139
Acetic acid (mmol L^{-1})	–	–	−0.563	0.090
Butyric acid (mmol L^{-1})	–	–	−0.448	0.194

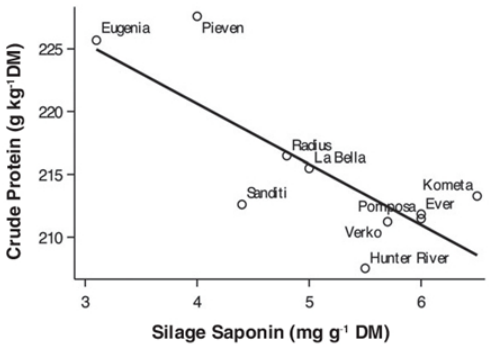

Figure 1. Linear relationship between protein (g kg^{-1} DM) and saponin content (mg g^{-1} DM); each data point represents one silage (protein (g kg^{-1} DM = 239.9–4.82 × saponin (mg g^{-1} DM).

AS ranged from 3.1 mg g^{-1} DM in Eugenia to 6.5 mg g^{-1} DM in Kometa. The average increase for the ten varieties was 2.7-fold, although this differed markedly for other varieties. The greatest increase (4.4-fold) was seen in Eugenia, which also had the lowest saponin concentration in the fresh sample. In general, varieties with a lower saponin concentration in fresh samples lead to greater increases in saponin content through ensiling.

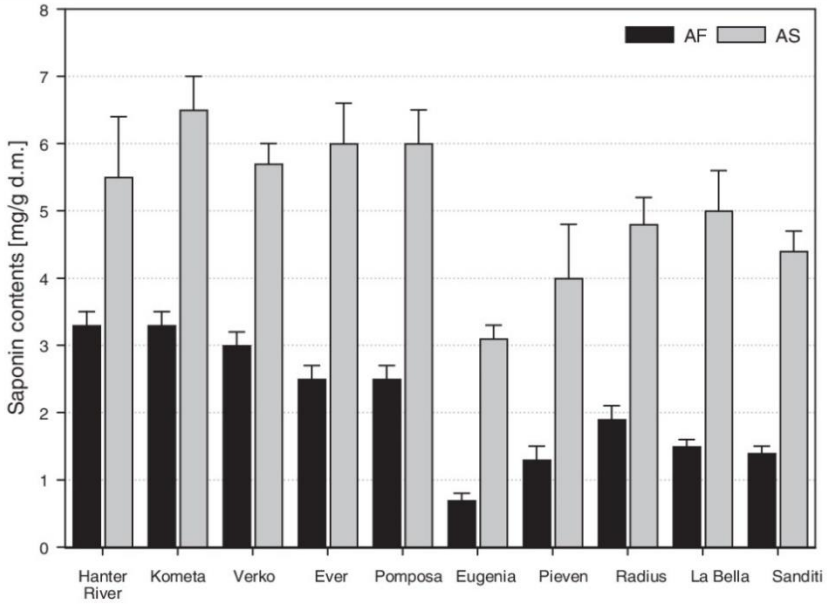

Figure 2. Total saponin content in AF (form of green feed for grazing) and AS (ensiled alfalfa).

Changes in saponin composition during ensiling

In the present study, we monitored five zanhic acid glycosides in AF and AS (Fig. 3). Three of these, ZATA, ZATB and ZAU, degraded substantially during ensiling. The other two (ZAGA and ZAG) dramatically increased in all varieties after ensiling. Of the seven medicagenic acid glycosides that could be identified based on the available standards, the concentration of five of these (MAA, MAB, MAC, MAG2 and MAMG) did not substantially change after ensiling, whereas the other two (MAGA and MAG1) almost totally disappeared in the silaged samples. Two glycosides of soyasapogenol B behaved differently: the glycoside SSI decreased, whereas SSII increased at the same time.

Another important group of alfalfa saponins are the glycosides of zanhic acid; five ZA glycosides were identified, including bisdesmosidic and tridesmosidic forms. The concentrations of the two soyasapogenol B derivatives observed in AF (SSI and SSII) were altered in different ways during ensiling. The total concentration of saponins increased two- to three-fold after ensiling compared to the fresh alfalfa material.

DISCUSSION

Chemical composition of raw and ensilaged alfalfa

Ensiling of alfalfa alone is typically avoided because of processing problems,[1] including the low water-soluble carbohydrate content, high buffer capacity, low pH[2] and extensive proteolysis during ensiling, which lowers the quality of the resulting silage. Thus, ensiling alfalfa with other plant species, such as corn[12] or sweet sorghum,[13] is a reasonable strategy for producing good quality silage with increased CP content. Nevertheless, for the present study, it was decided to ensile alfalfa alone to trace the fate of the saponins during the ensiling process. However, to ensure that the ensiling process was performed properly, we analyzed the chemical composition of both AF and AS. The results obtained for DM (294–364 g kg^{-1}) and CP (207–228 g kg^{-1}) are in line with those reported previously,[14–16] and confirm the dynamic pattern of fermentation and the microbial characteristics of alfalfa silage.

Higher concentrations of ammonia (approximately 5% to 7% of total N) were observed in the ensiled alfalfa. This may be caused by the rate of proteolysis after harvesting and silage storage.[17] Increased proteolysis increases the concentration of ammonia-N.[18] However, other factors, such as the additives used, may also affect this.[19,20] Our results are fairly high but nonetheless are in line with those of previous studies.[21,22]

We calculated the Pearson correlation coefficients between the saponin content (mg g^{-1} DM) and the chemical compositions of the silages and fresh material, confirming that the processes occurring during ensiling change both saponin content and protein concentration.

Total saponin concentration

The concentration of saponins in the aerial parts of alfalfa has been extensively researched. Depending on the method of determination, the alfalfa variety, the sampling date, the season, and the environmental conditions, the results have varied greatly.[23] Biological assay methods based on hemolysis or *Trichoderma viride* tests have usually resulted in a higher saponin concentration, ranging between 5 and 15 mg g^{-1} DM. Analytical methods based on liquid chromatography usually give lower values, probably as a result of incomplete extraction with solvents or differences in the biological activity of individual saponins.[24] One study found the saponin concentration of the Hodoninka and Palava varieties to be 1.68 mg g^{-1} DM and 2.41 mg g^{-1} DM, respectively.[11] Similarly, the concentration of saponins measured by liquid chromatography with a mass detector in three subspecies of *Medicago truncatula* ranged between 1.53 and 2.23 mg g^{-1} DM. These results correspond well with the concentrations of saponins obtained from the samples of alfalfa in the present study.

The findings of present study are in apparent disagreement with the data obtained by Kalac *et al.*,[24] who reported a decrease in

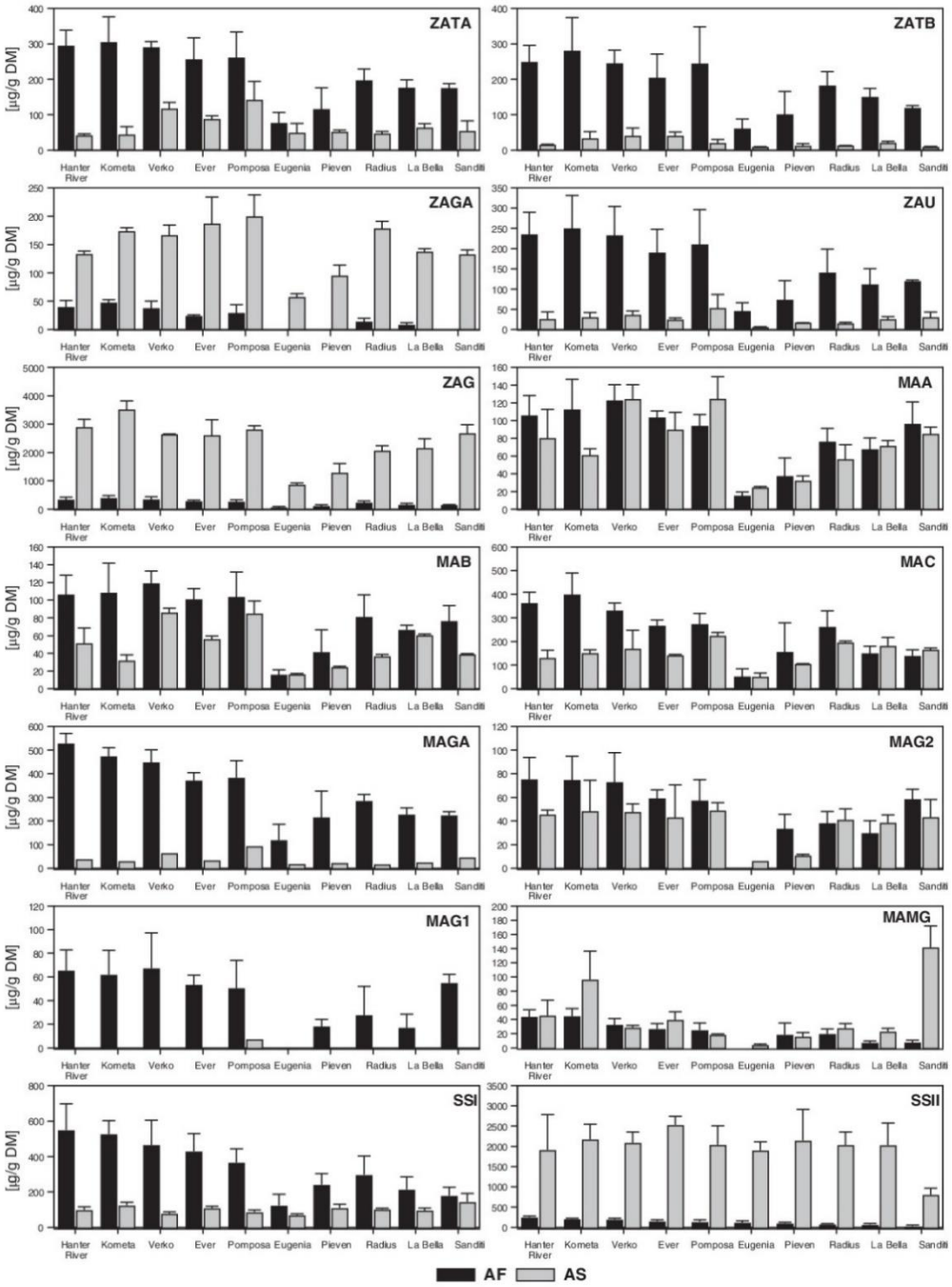

Figure 3. Changes in individual saponins during alfalfa ensiling. AF: form of green feed for grazing. AS: ensiled alfalfa.

saponin content in two alfalfa varieties during ensiling; however, the losses were not the same for structurally different individual saponins. It was concluded that enzymatic hydrolysis takes place during ensiling, although Kalac *et al.*,[24] were unable to detect any MA or ZA aglycones in the samples. Moreover, it is suggested that progress in understanding saponin behavior during ensiling will only be possible with further advances in analytical methodology.

However, reported abnormal increases in saponin content were also reported during a prolonged (7–30 days) ensiling processes. Two different hypotheses might explain this: the first is related to the loss of biomass during the ensiling process[25]; in 30 days

of alfalfa ensiling, over 12% of its DM is lost,[26,27] typically in the form of effluents and gases. In our case, there were no effluents, although the DM of AS increased substantially in all ten varieties.[28–30] A second explanation for the substantial increase in AS saponin content could be the better availability of chemicals in the ensiled samples. Saponins have an affinity to plant and animal membranes. They can form quite stable complexes with membrane sterols and are not easily extractable with organic solvents. Enzymatic activities during ensiling may release these bindings and make saponins more readily available.

Changes in saponin composition during ensiling

Although the total saponin content has been used as a parameter indicating the quality of forage, because of the different biological activities expressed,[6,23,30] few studies have been performed on pure individual compounds, on account of the extreme difficulties in separating these highly polar compounds.[23] Our previous studies of individual compounds have found large differences between individual saponins, depending on their chemical structures. These studies have revealed that the most important of these from the nutritional perspective, as well as the most biologically active, are the glycosides of medicagenic acid.[4,6]

Because of our results, it was of great interest to determine the fate of MA glycosides during ensiling, considering that the research of Kalac *et al.*[24] reported that the dominant compound of this group present in AF was almost completely degraded over 90 days. The standards available to use and the use of mass spectrometry data made it possible in the present study to identify at least seven MA glycosides in AF and AS (Fig. 3). Our results show that the majority of glycosides were chemically restructured (hydrolyzed or oxidized) in different ways. It is worth noting the structural similarities of MAGA and MAG1, which possess identical sugar-chains at position C28 on medicagenic acid, with the only difference being the substitution of glucuronic acid at C3 in MAGA and glucose in MAG1.

The glycosides of zanhic acid, another important group of saponins, remained undetected in alfalfa for a long time. The most abundant glycosides are very polar and were not seen with classical analytical methods. Because they are less active in traditional biological tests (*T. viride* and hemolysis), they were not recognized as crucial for fodder quality.[5] However, as shown with human volunteers, they are the most bitter and most throat-irritating compounds of all the tested alfalfa saponins. If the taste reaction in animals is similar, the palatability of an alfalfa-based diet may be lowered and may adversely affect feed intake.[3] Feed intake is the primary mechanism by which legume saponins exert their growth-depressing effects.[23] Once swallowed, ZA glycosides can cause intestinal discomfort, especially in monogastric animals. For this reason, ZA glycosides in AF and AS should also be carefully considered. In the present study, five ZA glycosides were identified, including bisdesmosidic and tridesmosidic forms. Again, similar to MA glycosides, three of these compounds (ZATA, ZATB and ZAU) were hydrolyzed during ensiling, and some were found in trace amounts in AS at the end of the experiment. This result agrees with studies reporting a progressive decrease in ZA tridesmoside level during alfalfa ensiling. However, our results show that, at the same time, the concentration of bisdesmosidic forms (ZAGA and ZAG) increased from trace levels in AF to relatively large amounts in AS. Because these compounds were not previously isolated in pure form, no conclusions can be made about their nutritional significance. ZAG is found in a significant amount in AS, with ZAGA found in a lower concentration, although both these compounds need to be isolated from AS so that their biological activities can be determined. It appears that these particular structures are very resistant to the enzymatic processes that occur during ensiling, and more attention should be paid to their nutritional significance. It is worth noting the similarities between the pairs of compounds, MAGA *versus* ZAGA and ZAG *versus* MAG1; they possess identical sugar chains in the saponin moiety and differ in their aglycone structures with respect to medicagenic acid in MAGA and MAG1, as well as zanhic acid in ZAGA and ZAG. If we consider that the difference between MA and ZA is only the substitution at carbon C18 hydrogen in MA and a hydroxyl group in ZA, it is highly probable that, during ensiling, MAGA and MAG1 are oxidized at carbon C18 to form ZAGA and ZAG. For this reason, MAGA and MAG1 disappear during ensiling, whereas ZAGA and ZAG increase; however, this mechanism needs to be demonstrated.

The third group of saponins includes two glycosides of soyasapogenol B that widely occur in legumes.[23,30] In many biological tests, these glycosides do not show significant activity and do not appear to be very important from a nutritional point of view. Interestingly, the concentrations of the two soyasapogenol B derivatives observed in AF (SSI and SSII) change in different ways during ensiling. These two compounds differed only in one carbohydrate residue (SSII has galactose, whereas SSI has xylose in the middle of the trisaccharide side-chain). It was surprising to find that SSI substantially dominates in AF, whereas, in AS, SSI is found only in trace amounts and SSII is present in very high amounts. This conversion of SSI to SSII during ensiling is difficult to interpret at the moment but, to our knowledge, no such transformation has previously been described.

In conclusion, it should be emphasized that the ensiling process of fodder crops containing saponins must be researched with great care. The simple determination of the saponins for which analytical standards are available is not sufficient to fully characterize silage quality. These compounds, similar to others (such as phenolics), undergo chemical transformation; to obtain a full understanding of their content and structural diversity, careful analysis with sophisticated analytical procedures must be employed.

Chemical analysis of AF and AS indicates that the total concentration of saponins increases two- to three-fold after ensiling compared to fresh alfalfa material. These changes are associated with a linear relationship between protein (g kg^{-1} DM) and saponin levels. Also, the transformation processes that occur during ensiling involve the transformation of some compounds to others that were either absent from the fresh material or occurred only in trace amounts. Isolating these compounds and investigating their biological activities is fundamental to determining the quality of alfalfa-derived silage feedstuff.

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Research Article

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The effect of total and individual alfalfa saponins on rumen methane production

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Abstract

BACKGROUND: Ten varieties of alfalfa (*Medicago sativa* L.) were evaluated for saponin content. Two of the most promising varieties were chosen so that their effect on rumen fermentation and methane production could be studied. Initially, four Hohenheim gas tests (HGT) were performed to test the effect of increased levels of total saponin extracted from the two alfalfa cultivars (Kometa and Verko) – either as fresh material or ensiled – on the total bacteria, total protozoa, methane emission, and selected methanogenic population. Afterwards, seven particular saponins were extracted from fresh alfalfa of the Kometa variety and tested in 24 h batch fermentation culture experiments.

RESULTS: The ensiled forms of both the Verko and Kometa alfalfa varieties seem to be good sources of saponin, capable of reducing methane production ($P < 0.05$) without negatively affecting the basic fermentation parameters. Of the two evaluated varieties, Kometa was the most effective, and the saponins extracted from its roots 3-Glc,28-Glc Ma, medicagenic saponin, and 3-Glu Ma showed the most evident effect ($P = 0.0001$). The most promising aerial alfalfa saponin in mitigating methane production was soysaponin I K salt ($P = 0.0001$). Three mixtures of saponins were tested and all were found to mitigate methane production; however, one mixture (MIX 1) did so only to a very small extent.

CONCLUSION: Saponins have been observed to have differing effects depending on their source; however, the mode of action of saponins depends on their direct or probable indirect effect on the microorganisms involved in methane production.

Supporting information may be found in the online version of this article.

Keywords: alfalfa; saponin; Hohenheim; *in vitro*; rumen; methane

INTRODUCTION

Methane and carbon dioxide are natural gases produced in the rumen during fermentation by the microbial community. Methane emission is a key factor in climate change, as it exerts a global warming potential 28 times greater than that of CO_2.[1] Furthermore, methane produced by ruminant digestion results in significant dietary energy loss.[2] Developing strategies to mitigate methane production in dairy cows is a challenge in both environmental and economic terms. Antibiotics have been widely used as a supplement in the ruminant diet; their ability to modulate rumen fermentation and mitigate methane emission has been known for years.[3] However, in 2003, the European Commission banned the use of chemical supplements in ruminant nutrition (Directive 1831/2003/CEE), as excessive use of antibiotics could lead to bacteria developing resistance to the drugs, threatening human health.[3] This radical change in the law resulted in intense research into effective natural compounds that can inhibit enteric greenhouse gas production.

Saponins, which are plant secondary metabolites, have the ability to modulate rumen fermentation while reducing methane production and ammonia concentration, by modulating ruminal microbial populations, especially, of protozoa.[4–6] Saponins act against protozoa by reducing the integrity of their cell membranes.[7,8] Saponin extracts, whether from tropical plants (like *Yucca schidigera*, *Quillaja saponaria*, *Sapindus saponaria*, *Garcinia mangostana*, *Gliricidia sepium*, *Enterolobium cyclocarpum*, and

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Hedara helix) or from plants not previously used in ruminant nutrition have been widely used in research.[5,9,10]

Alfalfa (*Medicago sativa* L.) is rich in secondary metabolites, especially saponins. It also has high nutritive value, reasonable protein quality and high protein content, which makes it good forage for high-producing dairy cows.[11,12] This plant has increasingly been used in regions other than Europe. In Asia, alfalfa has recently been employed to address the shortage of crude protein in feed and to meet the production requirements for growing and lactating ruminants.[13] Ensiling alfalfa and by-products of alfalfa processing is also currently increasingly popular worldwide.[12,14] However, the effect of saponin extract powder from fresh and ensiled alfalfa on methane production has not been explored previously.

We hypothesize that saponins from different alfalfa varieties inhibit ruminal microbial populations, mostly protozoa, and thus reduce methane production. We also hypothesize that the source (different varieties) and the type (aerial, roots, or mixture) of individual saponins determine their effectiveness in methane mitigation.

The objectives of the present study were to explore the effect of total saponins extracted from two different alfalfa varieties, in fresh and ensiled form, on methane production and total microbial populations (as determined with the Hohenheim gas test), as well as the effect of individual saponins extracted from one alfalfa variety on the methane production and total microbial populations (using a batch culture *in vitro* model).

MATERIAL AND METHODS

The chemical compositions of the 10 varieties of alfalfa tested in this study has been described by Szumacher-Strabel *et al.*,[15] who analyzed the levels of individual saponins in fresh and ensiled alfalfa. In the present study, 10 alfalfa varieties (Verko, Kometa, Hunter River, Ever, Pomposa, Eugenia, Pieven, Radius, La Bella Campagnola, and Sanditi), in fresh and ensiled forms, were analyzed for their total saponins content (Table 1), with the aim of selecting the two varieties with the highest total saponins.

Four Hohenheim gas tests (HGT) were then performed to determine the effect of total saponins supplemented at different doses from the two selected alfalfa cultivars (Kometa and Verko). We tested the effects of dried fresh and ensiled alfalfa (the control groups) and of dried fresh and ensiled alfalfa, with two to four times the amount of saponin content as in the control groups, on rumen fermentation characteristics and methane production (Table 2).

Afterwards, a batch culture fermentation experiment was conducted. Fresh Kometa alfalfa was chosen for this experiment because of the very promising effect on methane mitigation shown in the HGT experiments (four times the amount of saponin content present in fresh Kometa alfalfa; 4.84 mg 400 g^{-1} DM; Table 2). We tested the effects of seven particular saponins extracted from the aerial and root parts of fresh Kometa alfalfa, administered individually or as a mixture (MIX1: saponins from leaves; MIX2: saponins from roots; MIX3: total saponins), on the microbial population, rumen fermentation, and methane production.

Collection and preparation of alfalfa varieties

As described in Szumacher-Strabel *et al.*,[15] seeds of 10 alfalfa varieties were purchased at a local market in Poland. A 100 m^2 plot of each variety was grown in experimental fields at Poznań University of Life Sciences in 2014. All details of the preparation of the alfalfa have been described in Szumacher-Strabel *et al.*[15]

Saponin extraction

Extraction of total and individual saponins from alfalfa aerial and root parts

The aerial and root parts of the fresh and ensiled alfalfa were lyophilized using a Christ Gamma 2–16 LSC freeze-drier (Martin Christ GmbH, Osterode am Harz, DE), and then ground and defatted with chloroform using a Soxhlet extractor (FOSS, Hillerød, DK). Quantitative analysis and determination of the saponins were performed on an Aquity UPLC system (Waters, Milford, MA, USA) equipped with a triple quadruple mass spectrometer (TQD, Waters, Milford, MA, USA), as described by Szumacher-Strabel *et al.*[15]

Experimental design and management

Hohenheim gas test

Four Hohenheim gas test (HGT) experiments were conducted, following Cieslak *et al.*[16] The control substrate consisted of 400 mg of dried fresh (Exp. 1 with Verko variety, Exp. 3 with Kometa variety) or ensiled (Exp. 2 with Verko variety, Exp. 4 with Kometa variety) alfalfa on a DM basis; the experimental group consisted of dried fresh or ensiled alfalfa enriched with total saponin extract powder to obtain two or four times higher saponin levels than in the dried fresh or ensiled alfalfa. Each

Table 1. Saponin content of different varieties of fresh and ensiled alfalfa

	Saponin content (mg g^{-1} DM)			
Variety	Silage (S)	Fresh (F)	Differences	S:F ratio
Verko	10.4	3.30	7.1	3.2
Kometa	10.3	3.02	7.3	3.4
Hunter River	8.90	3.26	5.6	2.7
Ever	9.12	2.54	6.6	3.6
Pomposa	9.43	2.52	6.9	3.7
Eugenia	4.50	0.71	3.8	6.3
Pieven	6.23	1.28	5.0	4.9
Radius	9.03	1.94	7.1	4.7
La Bella Campagnola	8.73	1.46	7.3	6.0
Sanditi	8.61	1.44	7.2	6.0

Table 2. Experimental treatment and saponin concentration of each HGT experiment

Experiment	Saponin content (mg 400 g^{-1} DM) Control group	2 ×	4 ×
Fresh Verko saponins (FV; Exp. 1)	FV1	FV2	FV4
	1.32	2.64	5.28
Ensiled Verko saponins (SV; Exp. 2)	SV1	SV2	SV4
	4.16	8.32	16.64
Fresh Kometa saponins (FK; Exp. 3)	FK1	FK2	FK4
	1.21	2.42	4.84
Ensiled Kometa saponins (SK; Exp. 4)	SK1	SK2	SK4
	4.12	8.24	16.48

treatment consisted of five Hohenheim 100 mL glass syringes (Häberle LABORTECHNIK GmbH+Co., Lonsee-Ettlenschieß, DE). Each experiment lasted 48 h and was repeated three times.

Preparation of inoculum and incubation

Fresh ruminal fluid was collected in a slaughterhouse from nine Polish Holstein–Friesian dairy cows (each repetition from three cows) fed 20 kg day^{-1} of DM of a 600:400 forage: concentrate. The ruminal fluid was filtered through a four-layers cheesecloth into two Schott Duran bottles (Schott North America, Elmsford, NY, USA) maintained at 39 °C under anaerobic conditions. They were immediately transported to the laboratory, placed in a water bath at 39 °C, and mixed in a beaker before being diluted with an artificial buffer solution, following a modified protocol of Cieslak *et al.*[16]

Batch fermentation culture experiment

The batch fermentation culture experiment (Exp. 5) was conducted following Cieslak *et al.*[16] Due to the small amount of extracted saponins from the fresh Kometa variety, half of the usual amounts of inoculum and substrates were used (20 mL of inoculum, 200 mg of substrate). Eleven treatments were prepared as follows: the control substrate (CON), consisting of 120 mg cellulose, 42 mg starch, and 38 mg gluten; ALS1, consisting of CON plus 2.4 mg of 3GLcA,28AraRhaXyl saponin from alfalfa leaves; ALS2, consisting of CON plus 2.4 mg of zanhic acid tridesmoside saponin from alfalfa leaves; ALS3, consisting of CON plus 2.4 mg of soysaponin I K salt from alfalfa leaves; ALS4, consisting of CON plus 2.4 mg of medicoside J saponin from alfalfa leaves; ARS1, consisting of CON plus 2.4 mg of3-Glc,28-Glc Ma saponin from alfalfa roots; ARS2, consisting of CON plus 2.4 mg of medicagenic saponin from alfalfa roots; ARS3, consisting of CON plus 2.4 mg of 3- Glu Ma saponin from alfalfa roots; MIX1, consisting of CON plus 2.4 mg of alfalfa aerial saponin mixture (0.6 mg of each saponin); MIX2, consisting of CON plus 2.4 mg of alfalfa root saponin mixture (0.8 mg of each saponin); and MIX3, consisting of CON plus 2.4 mg of total aerial and root alfalfa saponin mixture (0.34 of each saponin). The quantity of 2.4 mg of supplemented saponins was calculated based on the amount of total saponins in the fresh Kometa alfalfa multiplied by four and adjusted to the amounts of incubated DM (4.84 mg*200 mg DM 400 g^{-1} DM = 2.42 mg).

Sampling and chemical analysis

In the HGT experiments, total gas production was measured every 2 h during a 48 h incubation period, and then combined and presented as one volume. Methane concentration was analyzed twice, after 24 h and 48 h of incubation. The total gas production and methane concentrations were measured once in the batch culture experiment after 24 h of incubation. In all experiments, the concentration of methane in the headspace gas samples was quantified using a gas chromatograph (GC) (SRI Peak Simple model 310 Alltech, Torrance, CA, USA). The GC was equipped with a thermal conductivity detector and a Carboxen 1000 column (mesh side 60/80, 15 FT × 1.8 INS.S, Supelco, Bellefonte, PA, USA), in line with the protocol described by Cieslak *et al.*[16] The pH value of the rumen samples was measured immediately after sample collection using a type CP-104 pH meter. The ammonia concentration was determined using the colorimetric Nessler method. Protozoa counts were conducted under a light microscope (Primo Star 5, Zeiss, Jena, Germany) using a drop of buffered rumen fluid of a defined volume (10 µL for entodiniomorpha and 100 µL for holotrichs). The *in vitro* DM digestibility (IVDMD) was measured by transferring the fermented content of the incubation vessel (syringe or serum bottle) into a 50 mL Falcon tube, which had been previously weighed and washed with 50 mL of deionized water. The Falcon tube was centrifuged for 10 min at 3500×*g* and 4 °C and the supernatant was discarded. The Falcon was then dried at 50 °C for 4 days, transferred into a previously weighed glass crucible, and dried at 103 °C for 1 d. The percentage loss in weight of the incubated substrate DM after correction for the DM residue in the blank control was taken as IVDMD. Methanogens and total bacteria were quantified by fluorescence *in situ* hybridization (FISH). Briefly, 50 µL of the rumen fluid was diluted in phosphate-buffered saline (PBS) and pipetted onto 0.22 µm polycarbonate filters (Frisenette K02BP02500, Knebel, DK) and vacuumed (Vacuum KNF Vacuport, Neuberger, Witney, UK). After vacuuming, the filters were transferred onto cellulose disks for dehydration in an ethanol series (50%, 80%, and 90%; 3 min each). For each sample, a series of identical filters were used to determine optimal hybridization. Hybridization was carried out in 50 µL of hybridization buffer (0.9 mol L^{-1} NaCl; 20 mM Tris/HCl, pH 7.2; 0.01% SDS) containing oligonucleotide probes (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). After hybridization, the filters were washed with washing buffer (20 mM Tris/HCl, pH 7.2; 0.01% SDS; 5 mM EDTA) for 20 min at 48 °C. The filters were rinsed gently in distilled water, air-dried, and mounted on object glasses with VectaShield antifading agent (H-1000, Vector Laboratories Ltd, Peterborough, UK) containing DAPI (4′,6-diamidino-2-phenylindole). To distinguish the total count of bacteria (DAPI) from other methanogens in the rumen fluid, filters were maintained at 4 °C for 1 h in the dark before visualization using an Axio Imager M2 microscope (CarlZeiss Iberia, Madrid, Spain). Each of these methods was conducted in line with Cieślak *et al.*,[16] with some modifications.

Statistical analysis

The statistical analysis followed a randomized complete block design based on the above model:

Table 3. Effect of saponin extract powder from fresh alfalfa (Verko) on rumen fermentation parameters, methane concentration, and methanogen population (Exp. 1)

	Treatment[2†]				Contrast		*P* value		
Item	FV1	FV2	FV4	SEM	L	Q	T	R	T × R
pH	6.57	6.59	6.59	0.005	0.177	0.726	0.125	0.215	0.021
Bacteria ($\times 10^9$ mL^{-1})	1.82^c	1.92^b	2.15^a	0.043	0.005	0.216	0.0002	0.994	0.969
Protozoa ($\times 10^5$ mL^{-1})	93.5^a	80.9^{ab}	73.6^b	3.050	0.003	0.543	0.026	0.744	0.545
IVDMD (%)	69.2	69.9	69.6	0.916	0.876	0.812	0.966	0.369	0.856
NH_3 (mM)	22.4	20.1	20.3	0.534	0.100	0.248	0.174	0.193	0.756
TGP (mL)	93.7	89.5	90.0	1.323	0.275	0.418	0.391	0.429	0.309
CH_4 24 h (mM)	8.01	7.86	6.03	0.686	0.276	0.585	0.421	0.254	0.274
CH_4 48 h (mM)	21.6	21.2	18.2	0.720	0.054	0.346	0.067	0.319	0.121
CH_4 48 h/TGP (mM mL^{-1})	0.24^a	0.24^{ab}	0.19^b	0.009	0.018	0.173	0.037	0.174	0.434
CH_4 48 h/IVDMD (mM 100 g^{-1})	0.31	0.30	0.27	0.011	0.126	0.533	0.289	0.777	0.366
Methanogens									
Methanobacteriales ($\times 10^7$ mL^{-1})	0.46^a	0.33^b	0.25^c	0.026	0.014	0.544	0.0002	0.997	0.990
Methanomicrobiales ($\times 10^7$ mL^{-1})	0.49^a	0,33^b	0.25^c	0.030	<0.001	0.051	<0.0001	0.884	0.992
Total methanogens ($\times 10^8$ mL^{-1})	0.62^a	0.47^b	0.36^c	0.032	0.001	0.153	<0.0001	0.950	0.977

[†]Treatments: FV1: 400 mg of dried fresh alfalfa Verko; FV2: with twice as in FV1 the saponin content; FV4: with four times as in FV1 the saponin content. Values within rows with different lowercase letters are significantly different ($P < 0.05$). Contrasts: significance of linear (L) and quadratic (Q) components of the response to the supplemented levels of saponin extract, probability of significant effect due to the treatment (T), run (R), and their interaction (T × R).

$$Yij = \mu + Ti + Rj + \varepsilon ij$$

where *Yij* is any observation for which (*i*) is the treatment factor and (*j*) is the run factor; the treatment (*T*) in this model is fixed effects, while run (*R*) and the run by treatment interaction (εij) are random effects. Data were analyzed using a two-way ANOVA with Tukey's range test. However, in the batch culture experiment, we found no effect of experimental run on the results obtained, which is why it was excluded from the model and all data were re-run using a one-way ANOVA. Polynomial contrasts were used to describe linear and quadratic responses to the level of supplementation. In all analyses, significant effects were declared at $P < 0.05$. Significant differences are labeled with differing superscripts (a, b, c). All analyses were performed using SAS software (version 9.3; SAS Institute Inc., Cary, NC, USA).

RESULTS

Hohenheim gas test (HGT) experiments

Tables 3–6 show the effects on rumen fermentation parameters of the dried fresh and ensiled alfalfa enriched with total saponin extract powder so as to obtain two or four times greater saponin levels than in the case of the dried fresh and ensiled alfalfa. The first experiment (Exp. 1; Table 3) clearly shows a linear reduction ($P = 0.003$) in total protozoa count as the saponin level increases. On the other hand, a linear increase ($P = 0.005$) in total bacteria correlates with an increase in the amount of saponins. All methanogens were linearly reduced ($P < 0.05$) by saponin doses, although this was not in line with most methane concentration results. As shown in Table 4 (Exp. 2), the increase in saponin from the ensiled Verko variety led to a linear decrease ($P < 0.005$) in total protozoa count, followed by a linear increase in total bacteria count ($P < 0.005$). The methane concentrations at both 24 h and 48 h after incubation were linearly reduced ($P < 0.05$) at the highest saponin level. The total gas production (TGP) volume declined linearly ($P = 0.010$) at two and four times the level of saponin, compared to SV1. The methanogen count in Exp. 2 followed the pattern seen for the methane concentration, with a reduction ($P < 0.05$) being observed in all methanogen counts with the increase in saponin concentration. Increased saponin in fresh Kometa alfalfa (Exp. 3 and 4; Tables 5 and 6) did lead to a linear decrease in protozoa levels and in all methanogen counts ($P = 0.002$, $P < 0.001$, $P = 0.003$, $P < 0.001$, respectively) as well as in methane concentration ($P < 0.05$), at both 24 h and 48 h. Similarly, the ensiled Kometa alfalfa supplemented with saponin extract powder (Exp. 4, Table 6) resulted in a linear reduction ($P < 0.05$) in total protozoa and in all methanogen counts, total gas production, and methane concentration at 48 h after incubation. However, increased saponin levels in the ensiled Kometa alfalfa did quadratically reduce the total protozoa count and total methanogens, as well as the Methanomicrobiales population. As shown in Tables 5 and 6 (Exp. 3 and 4), the increased levels of saponin from fresh and the ensiled Kometa variety led to an increase in total bacteria count ($P < 0.0001$). Generally, in the case of both fresh and ensiled Kometa alfalfa, the increased saponin level did not affect the *in vitro* dry matter digestibility.

Batch fermentation culture experiment

The effect of particular alfalfa saponins on rumen fermentation, methane concentration, bacteria, and methanogen population has never been studied before. Rumen fermentation parameters were relatively unchanged by individual saponins (Exp. 5; Table 7–10). *In vitro* dry-matter digestibility was not affected (Table 7). The lowest pH value was observed in the control group, and the highest pH was found in ALS1 ($P < 0.05$). Alfalfa leaf saponins (ALS1, ALS3), alfalfa root saponins, and the saponin mixtures all decreased methane production (MIX2, MIX3; Table 8). Significant differences were also observed in CH_4/IVDMD (mM g^{-1}), except for ALS3 and MIX3. The reduction in total protozoan, entodiniomorph, bacterium, total methanogen, Methanobacteriales, and Methanomicrobiales counts (Tables 9 and 10) were

Table 4. Effect of saponin extract powder from Verko alfalfa silage on rumen fermentation parameters, methane concentration, and methanogen population (Exp. 2)

	Treatment†				Contrast		*P* value		
Item	SV1	SV2	SV4	SEM	L	Q	T	R	T × R
pH	6.55	6.40	6.42	0.041	0.211	0.365	0.386	0.325	0.885
Bacteria ($\times 10^9$ mL^{-1})	1.93^{c}	2.07^{b}	2.35^{a}	0.055	0.002	0.143	<0.0001	0.787	0.941
Protozoa ($\times 10^5$ mL^{-1})	81.6^{a}	65.9^{b}	65.0^{b}	2.595	0.001	0.026	0.004	0.608	0.362
IVDMD (%)	65.1	62.8	62.6	1.920	0.093	0.343	0.081	0.151	0.417
NH_3 (mM)	24.4	24.9	25.6	0.642	0.491	0.954	0.799	0.892	0.414
TGP (mL)	140^{a}	133^{a}	117^{b}	3.920	0.010	0.485	0.004	0.038	0.026
CH_4 24 h (mM)	12.8^{a}	10.19^{ab}	9.40^{b}	0.616	0.047	0.064	0.049	0.752	0.251
CH_4 48 h (mM)	23.9^{a}	19.9^{b}	12.5^{c}	1.493	<0.001	0.127	0.0002	0.494	0.419
CH_4 48 h/TGP (mM mL^{-1})	0.17^{a}	0.15^{a}	0.11^{b}	0.009	<0.001	0.261	0.003	0.843	0.956
CH_4 48 h/IVDMD (mM 100 g^{-1})	0.37^{a}	0.38^{a}	0.24^{b}	0.021	<0.001	0.002	0.0009	0.710	0.297
Methanogens									
Methanobacteriales ($\times 10^7$ mL^{-1})	0.41^{a}	0.31^{b}	0.29^{b}	0.016	0.013	0.155	0.0002	0.863	0.989
Methanomicrobiales ($\times 10^7$ mL^{-1})	0.38^{a}	0.36^{ab}	0.28^{b}	0.013	0.020	0.321	0.0004	0.966	0.957
Total methanogens ($\times 10^8$ mL^{-1})	0.63^{a}	0.57^{ab}	0.43^{b}	0.026	0.014	0.327	0.0002	0.975	0.997

†Treatments: FV1: 400 mg of dried ensiled alfalfa Verko; FV2: with twice as in FV1 the saponin content; FV4: with four times as in FV1 the saponin content. Values within rows with different lowercase letters are significantly different ($P < 0.05$). Contrasts: significance of linear (L) and quadratic (Q) components of the response to the supplemented levels of saponin extract, probability of significant effect due to the treatment (T), run (R), and their interaction (T × R).

Table 5. Effect of saponin extract powder from fresh alfalfa (Kometa) on rumen fermentation parameters, methane concentration, and methanogen population (Exp. 3)

	Treatment†				Contrast		*P* value		
Item	FK1	FK2	FK4	SEM	L	Q	T	R	T × R
pH	6.68	6.70	6.64	0.016	0.283	0.233	0.182	0.159	0.168
Bacteria ($\times 10^9$ mL^{-1})	1.82^{c}	1.92^{b}	2.14^{a}	0.041	0.031	0.199	<0.0001	0.980	0.9853
Protozoa ($\times 10^5$ mL^{-1})	84.8^{a}	70.2a^{b}	62.4^{b}	3.351	0.002	0.454	0.017	0.645	0.530
IVDMD (%)	70.9	71.9	71.8	1.048	0.757	0.836	0.942	0.390	0.767
NH_3 (mM)	21.7	19.5	19.7	0.515	0.103	0.249	0.181	0.199	0.774
TGP (mL)	90.5	86.5	86.8	1.336	0.285	0.476	0.474	0.379	0.542
CH_4 24 h (mM)	6.80	6.75	5.37	0.174	0.045	0.176	0.558	0.180	0.201
CH_4 48 h (mM)	20.6	20.4	17.4	0.696	0.048	0.309	0.058	0.339	0.122
CH_4 48 h/TGP (mM mL^{-1})	0.23	0.24	0.20	0.008	0.132	0.133	0.026	0.166	0.303
CH_4 48 h/IVDMD (mM 100 g^{-1})	0.29	0.28	0.24	0.011	0.057	0.526	0.275	0.735	0.400
Methanogens									
Methanobacteriales ($\times 10^7$ mL^{-1})	0.46^{a}	0.33^{b}	0.25^{c}	0.026	<0.001	0.011	0.0003	0.856	0.967
Methanomicrobiales ($\times 10^7$ mL^{-1})	0.48^{a}	0.33^{b}	0.24^{c}	0.030	0.003	0.909	<0.0001	0.929	0.996
Total methanogens ($\times 10^8$ mL^{-1})	0.62^{a}	0.46^{b}	0.36^{c}	0.032	<0.001	0.975	<0.0001	0.946	0.986

†Treatments: FV1: 400 mg of dried fresh alfalfa Kometa; FV2: with twice as in FV1 the saponin content; FV4: with four times as in FV1 the saponin content. Values within rows with different lowercase letters are significantly different ($P < 0.05$). Contrasts: significance of linear (L) and quadratic (Q) components of the response to the supplemented levels of saponin extract, probability of significant effect due to the treatment (T), run (R), and their interaction (T × R).

accompanied by decreased methane production. Based on these results, we can state that all the root saponins had a strong antiprotozoal, and consequently antimethanogenic, effect. However, they differed (mostly numerically) in their effects on other fermentation parameters. Two of four leaf saponins also showed antiprotozoal and antimethanogenic activity. When served as a mixture, decreases in methane production and protozoa population were also observed; however, the decrease was marginal in the case of MIX1.

DISCUSSION

Hohenheim gas test experiments

According to the results shown in Tables 3–6, the ensiled form of both alfalfa varieties more effectively lowered methane production than did the fresh material. This could be attributed to the presence of higher saponin content in ensiled alfalfa than in fresh alfalfa on a dry matter basis. It should be noted that the ensiling process of alfalfa increased the saponin content by up to 7.3 mg g^{-1} DM. This has been caused differences in the results

Table 6. Effect of saponin extract powder from Kometa alfalfa silage on rumen fermentation parameters, methane concentration, and methanogen population (Exp. 4)

	Treatment[†]				Contrast		*P*-value		
Item	SK1	SK2	SK4	SEM	L	Q	T	R	T × R
pH	6.69	6.56	6.53	0.043	0.158	0.595	0.419	0.438	0.962
Bacteria ($\times 10^9$ mL^{-1})	1.93^{c}	2.08^{b}	2.35^{a}	0.053	0.611	0.292	<0.0001	0.657	0.920
Protozoa ($\times 10^5$ mL^{-1})	71.9^{a}	56.2^{b}	56.3^{b}	2.520	0.001	0.018	0.002	0.473	0.183
IVDMD (%)	66.6	64.9	64.3	1.838	0.331	0.542	0.099	0.083	0.693
NH_3 (mM)	23.8	24.2	24.9	0.634	0.521	0.927	0.817	0.890	0.384
TGP (mL)	137^{a}	129^{ab}	113^{b}	4.114	0.013	0.600	0.006	0.054	0.029
CH_4 24 h (mM)	11.9^{a}	9.53^{b}	8.54^{b}	0.596	0.060	0.051	0.059	0.705	0.340
CH_4 48 h (mM)	22.9^{a}	19.1^{b}	11.5^{c}	1.500	<0.001	0.111	0.0002	0.507	0.392
CH_4 48 h/TGP (mM mL^{-1})	0.17^{a}	0.15^{a}	0.10^{b}	0.009	<0.001	0.186	0.003	0.852	0.890
CH_4 48 h/IVDMD (mM 100 g^{-1})	0.35^{a}	0.35^{a}	0.21^{b}	0.021	<0.001	0.005	0.001	0.749	0.282
Methanogens									
Methanobacteriales ($\times 10^7$/mL^{-1})	0.42^{a}	0.32^{b}	0.29^{b}	0.017	0.004	0.907	0.0002	0.818	0.969
Methanomicrobiales ($\times 10^7$ mL^{-1})	0.38^{a}	0.36^{a}	0.28^{b}	0.014	0.008	0.070	0.0005	0.887	0.981
Total methanogens ($\times 10^8$ mL^{-1})	0.63^{a}	0.57^{b}	0.43^{c}	0.026	0.001	0.051	0.0001	0.986	0.982

[†]Treatments: FV1: 400 mg of dried ensiled alfalfa Kometa; FV2: with twice as in FV1 the saponin content; FV4: with four times as in FV1 the saponin content. Values within rows with different lowercase letters are significantly different ($P < 0.05$). Contrasts: significance of linear (L) and quadratic (Q) components of the response to the supplemented levels of saponin extract, probability of significant effect due to the treatment (T), run (R), and their interaction (T × R).

Table 7. Effect of individual saponins isolated from fresh Kometa alfalfa on rumen fermentation parameters (Exp. 5)

Item [a]	pH	NH_3 (mM)	IVDMD (%)	TGP (mL)
CON	6.05^{c}	26.2^{ab}	61.5^{ab}	72.5^{ab}
Alfalfa aerial saponins (ALS)				
ALS1	6.42^{a}	22.7^{b}	57.6^{bc}	68.0^{c}
ALS2	6.14^{bc}	24.5^{ab}	62.9^{a}	74.2^{a}
ALS3	6.12^{bc}	24.9^{ab}	63.1^{a}	70.3^{bc}
ALS4	6.11^{bc}	27.9^{a}	62.7^{ab}	74.1^{a}
Alfalfa root saponins (ARS)				
ARS1	6.13^{bc}	23.2^{b}	62.4^{ab}	72.2^{ab}
ARS2	6.14^{bc}	23.5^{b}	64.3^{a}	71.2^{ab}
ARS3	6.15^{b}	25.2^{ab}	61.0^{ab}	72.2^{ab}
Mixtures of saponins (MIX)				
MIX1	6.14^{bc}	24.4^{ab}	62.9^{a}	72.8^{ab}
MIX2	6.12^{bc}	26.9^{ab}	62.7^{ab}	73.4^{a}
MIX3	6.18^{b}	21.0^{c}	59.8^{ab}	71.2^{ab}
SEM	0.013	0.356	0.372	0.281
P-value	0.0001	0.0001	0.0070	0.0001

Values within rows with different lowercase letters are significantly different ($P < 0.05$).
[a] Item: particular saponin. ALS1: 3GLcA,28AraRhaXyl; ALS2: zanhic acid tridesmoside; ALS3: soysaponin I K salt; ALS4: medicoside J; ARS1: 3-Glc,28-Glc Ma; ARS2: medicagenic; ARS3: 3-Glu Ma; MIX1: ALS1, ALS2, ALS3, and ALS4; MIX2: ARS1, ARS2, and ARS3; MIX3: ALS1, ALS2, ALS3, ALS4, ARS1, ARS2, and ARS3.

obtained between the same varieties of alfalfa but different starting material (fresh or ensiled) for experiments (Exp. 1, Exp. 3 and Exp. 2, Exp. 4). In Exp. 1, the increased level of saponin from fresh Verko alfalfa was associated with a reduction ($P < 0.02$) in the methanogen and protozoa counts; however, methane concentration was not affected by any dose of saponin. Usually, the methanogenic population requires hydrogen as a substrate for methanogenesis, which is provided by the protozoa population.[17] Consequently, a decrease in the total protozoa count should negatively alter the methanogenic population and subsequently decrease the methane concentration as with main saponin-rich sources – *Quillaja saponaria*, *Camellia sineis*, and *Yucca schidigera*.[2] Most methanogens present in the rumen belong to the *Methanobacteriaceae* family (89% and 99% of methanogens present in the rumen fluid and protozoa fractions, respectively), and the washout of protozoa resulted in a 27% decrease in methanogen population.[17] For this reason, it is also worth mentioning the protozoa-associated methanogens (PAM); these are methanogens that

Table 8. Effect of individual saponins isolated from Kometa alfalfa on methane production (Exp. 5)

Item [a]	CH_4 (mM)	CH_4/IVDMD (mM g^{-1})	CH_4/TGP (mM mL^{-1})
CON	13.1^{a}	104^{a}	0.18^{a}
Alfalfa aerial saponins (ALS)			
ALS1	11.6^{bc}	92^{b}	0.17^{ab}
ALS2	12.0^{ab}	96^{a}	0.17^{ab}
ALS3	11.5^{bc}	105^{a}	0.17^{ab}
ALS4	12.7^{ab}	99^{a}	0.17^{ab}
Alfalfa root saponins (ARS)			
ARS1	11.4^{bc}	91^{b}	0.16^{bc}
ARS2	10.2^{c}	80^{bc}	0.14^{c}
ARS3	11.4^{bc}	94^{b}	0.16^{bc}
Mixtures of saponins (MIX)			
MIX1	11.9^{ab}	98^{a}	0.17^{ab}
MIX2	11.7^{bc}	93^{b}	0.16^{bc}
MIX3	11.4^{bc}	96^{a}	0.16^{bc}
SEM	0.132	0.243	0.002
P value	0.0001	0.0001	0.0001

Values within rows with different lowercase letters are significantly different ($P < 0.05$).
[a] Item: particular saponin. ALS1: 3GLcA,28AraRhaXyl; ALS2: zanhic acid tridesmoside; ALS3: soysaponin I K salt; ALS4: medicoside J; ARS1: 3-Glc,28-Glc Ma; ARS2: medicagenic; ARS3: 3-Glu Ma; MIX1: ALS1, ALS2, ALS3, and ALS4; MIX2: ARS1, ARS2, and ARS3; MIX3: ALS1, ALS2, ALS3, ALS4, ARS1, ARS2, and ARS3.

Table 9. Effect of individual saponins isolated from Kometa alfalfa on protozoa population (Exp. 5)

Item [a]	Total Protozoa (10^4 mL^{-1})	Entodiniomorpha (10^4 mL^{-1})	Holotrichs (10^4 mL^{-1})
CON	3.42^{a}	3.25^{a}	0.17^{ab}
Alfalfa aerial saponins (ALS)			
ALS1	2.29^{b}	2.15^{b}	0.14^{bc}
ALS2	3.19^{a}	3.04^{a}	0.15^{bc}
ALS3	2.29^{b}	2.13^{b}	0.16^{ab}
ALS4	3.31^{a}	3.16^{a}	0.15^{bc}
Alfalfa root saponins (ARS)			
ARS1	2.34^{b}	2.18^{b}	0.16^{ab}
ARS2	2.43^{b}	2.33^{b}	0.10^{c}
ARS3	2.65^{b}	2.47^{b}	0.21^{a}
Mixtures of saponins (MIX)			
MIX1	3.15^{a}	2.98^{a}	0.17^{ab}
MIX2	2.15^{b}	1.98^{b}	0.17^{ab}
MIX3	2.42^{b}	2.25^{b}	0.17^{ab}
SEM	0.046	0.044	0.005
P-	value	0.0001	0.0001
0.0001			

Values within rows with different lowercase letters are significantly different ($P < 0.05$).
[a] Item: particular saponin. ALS1: 3GLcA,28AraRhaXyl; ALS2: zanhic acid tridesmoside; ALS3: soysaponin I K salt; ALS4: medicoside J; ARS1: 3-Glc,28-Glc Ma; ARS2: medicagenic; ARS3: 3-Glu Ma; MIX1: ALS1, ALS2, ALS3, and ALS4; MIX2: ARS1, ARS2, and ARS3; MIX3: ALS1, ALS2, ALS3, ALS4, ARS1, ARS2, and ARS3.

have become endosymbionts of protozoa, which produce hydrogen by malate oxidization.[18] This relationship is thought to grant mutual benefits to both. About 16% of rumen protozoa contain methanogens in their cells.[18] This low percentage can be explained by the unstable nature of the relationship; it may suggest that protozoa do not only play the role of hydrogen donors for methanogens. Additional studies would be required to confirm this.[13] However, according to Patra and Yu,[19] the decrease in methane production due to saponins could be attributed precisely to decreases in abundance of archaea, due to the inhibition of protozoa. This was not confirmed in Exp 1, where methane concentration was not affected. This, in turn, is in line with Goel *et al.*,[20] who showed that a decrease in methanogens does not always correlate with a decrease in methane concentration. Similar results were obtained in studies of saponins and tannins from mangosteen peel powder. Although a reduction was obtained in the protozoa population, no change in methane production was observed.[9] As mentioned by Patra *et al.*,[18] research has shown that saponins are likely to have little direct effect on methanogens, although it is known that they inhibit protozoa in the rumen, reducing both hydrogen production and the population of PAM. The effect of saponins on protozoa is attributed to their ability to form complexes with cholesterol in the cell membrane, altering its permeability and provoking cell lysis.[21,22] On the other hand, the rumen is a complex ecosystem, so any analysis of the effect of saponins on methanogen populations should take into account not only the total population of methanogens but also individual orders and species.[22]

In Exp. 2, using an increased level of saponin from ensiled Verko alfalfa led to a decrease in methane concentration as a result of reduced protozoa and methanogen counts. Many studies have highlighted a decrease in methane concentration of up to 50% with increases in saponin level.[23] This has been confirmed in the *in vitro* study of Belanche *et al*,[10] where addition of ivy fruit extract to the diet reduced methane release by 40%. It is worth mentioning that when protozoa are partially inhibited by saponin-rich sources, predation intensity is reduced; in consequence, the intensity of the predation of rumen bacteria by protozoa decreases.[1,2] This is in line with our results, which show higher bacterial counts with increased saponin levels. The reason for this might be that the saponins found in this alfalfa variety have a stimulating effect on the growth of some cellulolytic bacteria.[4]

Batch fermentation culture experiment

Based on the most promising methane mitigation results using the saponin extract powder obtained from the fresh Kometa varieties (Exp. 3: up to 38% after 24 h and up to 15% after 48 h of incubation; Exp. 4: up to 28% after 24 h and up to 50% after 48 h of incubation), individual saponins from this alfalfa variety's aerial or root parts were subjected to a batch culture experiment (Exp. 5). Saponins have potent antiprotozoal activity due to their formation of complex sterols in protozoan cell membranes.[22,24] Significant differences were also observed in CH_4/IVDMD (mM/g), except for ALS3 and MIX3. The reduction in total protozoan, entodiniomorph, bacterium, total methanogen, Methanobacteriales, and Methanomicrobiales counts (Tables 9 and 10) was accompanied by decreased methane production. Based on these results, we can state that all the root saponins had a strong antiprotozoal, and consequently antimethanogenic, effect. However, they differed (mostly numerically) in their effects on other fermentation parameters. Two of four leaf saponins also showed

Table 10. Effect of individual saponins isolated from Kometa alfalfa on bacteria and methanogenic population (Exp. 5)

Item [a]	Bacteria (10^8 mL^{-1})	Total methanogens (10^7 mL^{-1})	*Methanobacteriales* (10^7 mL^{-1})	*Methanomicrobiales* (10^7 mL^{-1})
CON	14.04^{a}	2.62^{a}	1.20^{a}	1.27^{a}
Alfalfa aerial saponins (ALS)				
ALS1	10.27^{bcd}	1.45^{d}	0.27^{b}	1.20^{a}
ALS2	10.80^{bc}	2.20^{abcd}	0.77^{ab}	0.63^{b}
ALS3	8.09^{de}	1.42^{d}	0.56^{b}	1.10^{a}
ALS 4	8.35^{de}	2.43^{abc}	0.71^{ab}	0.27^{c}
Alfalfa root saponins (ARS)				
ARS1	7.53^{e}	1.82^{bcd}	0.63^{b}	0.60^{b}
ARS2	7.96^{e}	1.74^{bcd}	0.49^{b}	0.37^{bc}
ARS3	8.08^{e}	1.60^{cd}	0.65^{b}	0.84^{b}
Mixtures of saponins (MIX)				
MIX1	11.14^{bc}	2.14^{abcd}	0.94^{ab}	1.02^{ab}
MIX2	11.25^{b}	1.60^{cd}	0.80^{ab}	0.94^{b}
MIX3	11.16^{bc}	2.49^{ab}	0.83^{ab}	1.37^{a}
SEM	0.167	0.083	0.059	0.079
P value	0.01	0.01	0.02	0.02

Values within rows with different lowercase letters are significantly different ($P < 0.05$).
[a] Item: particular saponin. ALS1: 3GLcA,28AraRhaXyl; ALS2: zanhic acid tridesmoside; ALS3: soysaponin I K salt; ALS4: medicoside J; ARS1: 3-Glc,28-Glc Ma; ARS2: medicagenic; ARS3: 3-Glu Ma; MIX1: ALS1, ALS2, ALS3, and ALS4; MIX2: ARS1, ARS2, and ARS3; MIX3: ALS1, ALS2, ALS3, ALS4, ARS1, ARS2, and ARS3.

antiprotozoal and antimethanogenic activity. Our research confirmed the fact that saponins limit the number of protozoa and methanogens, and thus reduce the production of methane. Our research also allows us to conclude that saponins from various parts of alfalfa affect the same groups of protozoa in various ways. So far, the literature has shown that protozoa have different sensitivities to saponins due to the different compositions of sterols in their membranes.[25] The antiprotozoal action of saponins is also limited by rumen microorganisms, which cause them to deglycosylate to sapogenins.[6] It has been suggested that, for example, glycosidase-inhibiting iminosugars could be used to reduce the effects of bacteria on saponins in the treatment.[25] To some extent, saponins also exhibit bacteriolytic activity in the rumen. Our research, however, shows that saponins are antiprotozoal at lower concentrations, and that higher concentrations can suppress methanogens. As mentioned, saponins inhibit ruminal bacterial and limit the availability of H_2 for methanogenesis in the rumen, thereby reducing CH_4 production.[23] Our research has also confirmed that the various saponins affect the populations of microorganisms (protozoa, bacteria, and methanogens) in different ways and thus limit the production of methane. The lowest pH in the control group may be due to the lack of a microorganism-limiting factor, which is saponin. Microorganisms therefore had easier access to easily available substrates such as cellulose, gluten and starch, which resulted in faster pH reduction.

The present research indicates that the effectiveness of saponins depends on their source, type, and concentration in plant products, thus confirming earlier observations.[26] Moreover, on the basis of our results, we can conclude that saponins extracted from Kometa alfalfa leaves mitigate methane production, total protozoa, bacteria, and methanogen populations less strongly than saponins extracted from Kometa alfalfa roots. This may suggest that steroid saponins could be more effective in mitigating ruminal CH_4 emissions than triterpenoid saponins.[2] Saponins consist of a hydrophobic aglycone backbone linked to hydrophilic sugar chains.[27] The difference in sugar moieties between these sources may also explain their distinct activities. Accordingly, the biological activity of saponins depends on the nature, number, and sequence of sugars in their structures.[23] For instance, monodesmosidic saponins (those with a single sugar chain) are generally more active than bidesmosidic saponins (with two sugar chains).[28] Further, substituting a monosaccharide with another monosaccharide within the sugar chain can alter the biological activity of a saponin.[29] It is, however, quite difficult to understand fully the structure-activity relationships of saponins on account of the large structural diversity of these substances (in both the sapogenin and the sugar moiety), even within a single plant species.[2,30]

Alfalfa would be an inexpensive source of saponins added to feed, due to the global popularity of this forage compared to other saponin-rich sources. These studies have shown that alfalfa mitigates methane emissions without adversely affecting rumen fluid fermentation. Incorporation of alfalfa silage or isolated saponins in diets would probably be economically and nutritionally beneficial for dairy cows.

CONCLUSION

We conclude that the ensiled form of both the Verko and Kometa alfalfa varieties seems to be a good source of saponin, capable of reducing methane production, as a result of reduced protozoa and methanogen counts, without negatively affecting the basic fermentation parameters. The Kometa variety proved to be the most effective of the two, and the saponins extracted from its roots (3-Glc,28-Glc Ma, medicagenic, 3-Glu Ma) showed the most evident effects. The most promising aerial alfalfa saponin in mitigating methane production was Soysaponin I K salt. All the three tested mixtures of saponins were found to mitigate methane production; however, MIX 1 did so only to a very small extent.

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Effects of partially replacing grass silage by lucerne silage cultivars in a high-forage diet on ruminal fermentation, methane production, and fatty acid composition in the rumen and milk of dairy cows

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ABSTRACT

Lucerne silages of the Kometa and Verko cultivars were investigated in four consecutive experiments to evaluate their effects on ruminal fermentation and fatty acid (FA) proportions in the rumen fluid and milk of dairy cows. Initially, two successive in vitro experiments were performed using a semicontinuous culture system (RUSITEC). In Experiment 1, the treatments were: (1) MGS: a high-forage diet (65:35 forage:concentrate ratio; DM basis) containing maize silages (MS) and grass silages (GS) as the main forage sources, (2) MKLS: a high-forage diet containing MS and Kometa lucerne silage (KLS) as the main forage sources; and (3) sMKLS: a high-forage diet containing MS and KLS as the main forage sources and supplemented with sufficient saponin extract powder from KLS to give a saponin content equal to the in the KLS. The treatments in Experiment 2 (MGS, MVLS, or sMVLS) were similar to those in Experiment 1, except that Verko Lucerne silage (VLS) and its saponin extract were used. Methane concentration statistically insignificant decreased ($P = 0.97$) as a result of the MKLS treatment, while digestible dry matter (DDM) was increased ($P = 0.002$) for MKLS and sMKLS. However, neither methane concentration nor DDM were affected by MVLS or sMVLS. We also performed two in vivo experiments: in Experiment 3, we used six cannulated Polish Holstein-Friesian cows fitted with rumen cannulas, and in Experiment 4, we used 54 productive Polish Holstein-Friesian cows. The treatments for these two

Abbreviations: GS, grass silage; LS, lucerne silage; MS, maize silage; KLS, Kometa lucerne silage; VLS, Verko lucerne silage; MGS, a high-forage diet (65:35 forage:concentrate ratio: DM basis) containing MS and GS as the main forage sources; MKLS, a high-forage diet containing MS and KLS as the main forage sources; sMKLS, a high-forage diet containing MS and KLS as the main forage sources and supplemented with sufficient saponin extract powder from KLS to give a saponin content equal to the in the KLS; sVLS, a high-forage diet containing MS and VLS as the main forage sources; sMVLS, a high-forage diet containing MS and VLS as the main forage sources and supplemented with sufficient saponin extract powder from VLS to give a saponin content equal to the in the KLS; FA, fatty acids; VFA, volatile fatty acids.

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experiments were based on partial replacement of GS with KLS or VLS in the high-forage diet containing MS, as described earlier: MGS, MKLS, and MVLS. In Experiment 3, the methane concentration decreased ($P < 0.001$) for both MKLS and MVLS, with the diets lowering the total bacterial and protozoal counts and the total methanogens ($P \leq 0.002$). Interestingly in Experiment 4, only MVLS led to a decrease in fat content and energy ($P \leq 0.02$). MKLS and MVLS were also associated with lower levels of milk polyunsaturated fatty acid ($P < 0.001$) and higher levels of milk saturated fatty acid ($P < 0.001$). MKLS had more promising fatty acid composition than MVLS, with clearer methane mitigation. In conclusion, the Kometa lucerne silages inhibited methane emission and microbial population, but at the cost of increasing the saturated fatty acids in the milk fat.

1. Introduction

Plant extracts containing secondary metabolites have been extensively assessed for their potential to modulate ruminal fermentation and improve nutrient utilization in ruminants (Benchaar et al., 2007; Patra and Saxena, 2011). Saponins, which are plant secondary metabolites, inhibit rumen ciliate protozoa (Teferedegne, 2000; Benchaar et al., 2007) and often decrease rumen methanogenesis (Hess et al., 2003; Santoso et al., 2006). They are effective because about 25 % of ruminal methanogens live in association with protozoa (Newbold et al., 1997). Reducing methanogenesis helps increase bovine energy efficiency (Johnson and Johnson, 1995) and is suitable for the environment, as methane is a potent greenhouse gas (Intergovernmental Panel on Climate Change, 2007). *Medicago sativa* L., commonly known as lucerne or alfalfa, is one of the most valuable legume plants for ruminants that can be widely grown in a temperate climate. Lucerne is rich in saponins, mainly pentacyclic triterpenoids (Oleszek et al., 1990; Szumacher-Strabel et al., 2019). It has been shown that the total saponin concentration in lucerne silage (**LS**) is higher than in fresh lucerne as a result of the ensiling processes (Szumacher-Strabel et al., 2019). The transformation processes that occur during ensiling involve the conversion of some compounds to others and increase the availability of absent or occurred in slight amounts compounds in fresh material (Szumacher-Strabel et al., 2019), hence the proportion of fresh feed components is affected by the consumption and/or conversion of those compounds during silage preparation, consequently increasing the proportion/concentration of some other compounds in silage compared to the same weight of fresh material.

Besides, varieties of lucerne differ in their individual and total saponins content (Szumacher-Strabel et al., 2019). Kozłowska et al. (2020) showed that the Polish Kometa and German Verko cultivars have the greatest total saponins of lucerne varieties. Their ensiled forms can reduce methane production in cows in vitro study without negatively affecting the basic fermentation parameters (Kozłowska et al., 2020).

Interest in enriching ruminant-derived foods, such as meat and milk, with health-promoting fatty acids (**FA**) such as *cis*-9, *trans*-11 conjugated linoleic acid (**CLA**) and 18:3n-3 has been growing. Plant secondary metabolites such as saponins can also be considered as means to manipulate rumen microbiota (Wang et al., 2009) and perhaps modify the FA composition of milk and meat (Wina et al., 2005). Benchaar and Chouinard (2009) fed saponins from *Yucca schidigera* (60 g/d) to dairy cows and noted minor decreases in the proportions of milk C6:0, C8:0, and *trans*-11 C18:1. Szczechowiak et al. (2016) fed *Saponaria Officinalis* root (440 g/d) to dairy cows as a source of triterpenoid rich saponins and found increases in the concentrations of *cis*-9 18:1, *trans*-11 C18:1, *cis*-9, *cis*-12 C18:2, *cis*-9, *trans*-11 CLA in their milk. Wang et al. (2017) observed that feeding 40 g/day (g/d) of tea saponins to dairy cows increased the levels of unsaturated FA, including *cis*-9 18:1, in milk during the first 4 wk of feeding. Later the levels tended to revert to those of the milk of cows receiving the control diet. However, less work has been conducted on the potential effect of LS than on grass silage (GS). At least part of the enrichment in milk can be attributed to higher amounts of 18:3n-3 escaping the rumen (Dewhurst et al., 2003a, b).

In this study, we hypothesize that replacing GS with LS can modulate ruminal fermentation, lower methane production, and improve milk FA composition, at least partly due to the LS saponin content. Moreover, based on the previous study (Kozłowska et al., 2020) where more promising results regarding ruminal parameters as well as methane decreasing in case of Kometa variety were obtained, we also hypothesize that this response would be greater with Kometa LS (**KLS**) than with Verko LS (**VLS**). The objectives of this study were to examine the effects of partially replacing GS by KLS or VLS in a high-forage diet containing MS on in vitro ruminal fermentation using Rusitec fermenters, in vivo ruminal fermentation, ruminal FA composition, dry matter intake, and total-tract digestibility using rumen-cannulated cows, as well as on in vivo milk production and FA composition in commercial dairy cows.

2. Materials and methods

Four experiments were conducted in this study: two in vitro ruminal fermentation experiments (Experiments 1 and 2) at Poznań University of Life Sciences (Poznań, Poland) using the Rusitec equipment with three fermenters, and two in vivo experiments addressing ruminal fermentation (Experiment 3) and milk production performance (Experiment 4), which were carried out on a commercial dairy farm (Szemborowo, Poland) using six rumen-cannulated cows and 54 producing dairy cows, respectively. The cows were cared for following 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. 25/2012).

2.1. Experimental design, treatments, and management

2.1.1. In vitro experiments (Experiments 1 and 2)

The rumen simulation technique (Czerkawski and Breckenridge, 1977) was used to evaluate longer-term ruminal fermentation responses to experimental treatments. The Rusitec system was equipped with three fermenters (three incubation vessels), each with a volume of one liter. Each experiment was conducted with three incubation runs (blocks). In each run, the three fermenters (experimental units) were randomly assigned to one of the three treatments. Each run lasted ten days, with five-day adaptation periods and five-day sampling periods.

On day 1 of each run, each vessel was filled with 720 mL of pooled strained ruminal fluid and 100 mL of prewarmed artificial saliva (McDougall's buffer; McDougall, 1948). In addition, pooled ruminal solids (11 g DM) and one of the three experimental feed substrates (11 g DM) contained in two separate nylon bags (70 × 140 mm; 100 μm pore size; Benetex, Poznań, Poland) were inoculated into the perforated feed container inside of each vessel. On day 2, the bag containing the ruminal solids used as inoculum was removed and replaced with a bag containing the feed substrate. After that, until the end of each run, each feed bag incubated for 48 h was removed and replaced by a bag containing the feed substrate. Each nylon bag removed from the fermenter after 48 h of incubation was rinsed with 50 mL artificial saliva to recover the microbes adhering to feed particles. The artificial saliva used for rinsing was returned to the fermenter at each feeding time.

During each run, the fermenters were maintained at 39 °C in a water bath, mixed continuously at 15 rpm with an impeller stirrer, and purged with CO_2 gas (99 % purity). An electronic peristaltic pump (Miniplus 3; Gilson, Middleton, WI, USA) was employed to introduce the artificial saliva to each fermenter at a constant flow rate of 0.347 mL/min (500 mL of saliva/d). To halt fermentation in the effluent vessels, 10 mL of 10 M H_2SO_4 was added to each. To re-establish the anaerobic conditions in the gaseous phase of the fermenters after opening the system to change the bags, nitrogen gas was flushed into the system for 3 min at 3 L/min.

Fresh rumen inoculum for each incubation run was obtained from the pooled rumen content collected 3 h after the morning feeding from three rumen-cannulated multiparous Polish Holstein-Friesian dairy cows (612 ± 24 kg body weight, in the fourth month of

Table 1
Ingredients and chemical composition of experimental diets used in in vitro and in vivo experiments [1].

Item	Treatments [2]		
	MGS	MKLS	MVLS
Ingredient, g/kg DM			
Maize silage	371	384	384
Grass silage	234	82	82
Kometa lucerne silage	22	188	–
Verko lucerne silage	22	–	188
Beet pulp	106	110	110
Supplement 1 [3]	79	82	82
Brewer's grain	66	69	69
Extracted rapeseed meal	57	41	41
Supplement 2 [4]	31	32	32
Mineral-vitamin premix [5]	12	12	12
Chemical composition [6], g/kg DM			
DM, g/kg as fed	430	438	434
OM	908	915	921
aNDFom	336	317	336
CP	176	179	179
EE	25	32	30
Fatty acid composition, g/100 g total FA			
C14:0	0.74	0.69	0.51
C16:0	25.0	24.1	24.5
C16:1 *cis*-9	0.78	0.86	1.07
C18:0	3.90	3.55	3.21
C18:1 *cis*-9	17.5	16.2	16.0
C18:2 *cis*-9,*cis*-12	41.8	43.5	43.2
C18:3 *cis*-9,*cis*-12,*cis*-15	10.2	11.1	11.5

[1] In the in vitro experiments, the diets were as a total mixed ration (TMR). In the in vivo experiments, the diets were fed as a partial mixed ration (PMR) along with supplement 2 (as top-dress) at 2 kg/d/cow.

[2] MGS = a high-forage diet (65:35 forage:concentrate ratio; DM basis) containing maize silage (MS) and grass silage as the main forage sources; MKLS = a high-forage diet containing MS and Kometa lucerne silage as the main forage sources; MVLS = a high-forage diet containing MS and Verko lucerne silage as the main forage sources.

[3] Stated to contain (as g/kg of DM in concentrate) OM (910), aNDFom (240), CP (17.5), EE (31).

[4] Supplement 2 was fed as top-dress at 2 kg/d/cow in in vivo experiment. Stated to contain (as g/kg of DM in concentrate) OM (900), aNDFom (250), CP (21.5), EE (29).

[5] Stated to contain (g/kg of DM) Na (123), Ca (100), Mg (45), P (42), K (20), S (18), Co (14), Cu (5), Zn (2.8), Mn (1.4), Fe (1.1), F (0.42), I (0.03), Se (0.02), biotin (0.01); (IU/kg), vitamin A (200,000), vitamin D3 (40,000), and vitamin E (1200).

[6] OM = organic matter; aNDFom = ash free adjusted neutral detergent fiber; CP = crude protein; EE = ether extract.

lactation) fed a TMR (at 24.4 kg of DM/d) similar to the MGS diet presented in Table 1. The rumen contents from each cow (approximately 600 g) were collected from the top, bottom, and middle parts of the rumen. An equal amount of rumen content from all cows was mixed, strained through four layers of cheesecloth, put in a bottle flushed with nitrogen gas, and immediately transported under anaerobic condition in a water bath at 39 °C to the laboratory.

In Experiment 1, the treatments were: (1) **MGS**: a high-forage diet (65:35 forage:concentrate ratio; DM basis) containing MS (371 g/kg DM) and GS (234 g/kg DM) as the main forage sources, (2) **MKLS**: a high-forage diet containing MS (384 g/kg DM) and KLS (188 g/kg DM) as the main forage sources; and (3) **sMKLS**: a high-forage diet containing MS and KLS as the main forage sources and supplemented with sufficient saponin extract powder from KLS to give a saponin content equal to the in the KLS (Szumacher-Strabel et al., 2019; Kozłowska et al., 2019). The treatments in Experiment 2 (**MGS, MVLS,** or **sMVLS**) were similar to those in Experiment 1, except that VLS and its saponin extract were used. The ingredients and chemical composition of MGS, MKLS, and MVLS diets are given in Table 1. The MKLS and MVLS diets were prepared by partially replacing GS with KLS or VLS and slightly decreasing the proportion of extracted rapeseed meal to adjust dietary CP. The MGS, MKLS, and MVLS diets were also used in in vivo experiments; for this reason, all the diets were formulated using the FeedExpert software (Rovecom, Hoogeveen, Netherlands) to meet or exceed the nutrient requirements of cows in mid-lactation, producing 32 kg milk/d.

For incubation of the treatments in the fermenters, all the feedstuffs were dried separately at 55 °C for 24 h, milled to pass through a 1-mm screen to discard the fine particles that would be lost mechanically through the bag cloth (Arthur H. Thomas Philadelphia, PA, USA); and then mixed based on the DM basis amounts shown in Table 1. For each run, each fermenter was fed daily (at 11:00 am) with one of the three experimental feed substrates (11 g DM) in a nylon bag. For the sMKLS and sMVLS substrates, each nylon bag was fed with 1.89 g of dried LS [containing 0.11 mg of saponin (Szumacher-Strabel et al., 2019; Kozłowska et al., 2019)], 0.11 mg of saponin extract powder, and 9.11 g of other dried feedstuffs.

The saponin extract powder from KLS or VLS was prepared as described by Szumacher-Strabel et al. (2019). Briefly, the aerial and root parts of LS were separately lyophilized using a Christ Gamma 2–16 LSC freeze-drier (Martin Christ, Osterode am Harz, Germany), ground, and defatted with chloroform using a Soxhlet apparatus (FOSS, Hillerød, Denmark). Samples were extracted using a water-methanol solution in a round-bottom flask under reflux. After filtering, the extract was evaporated under reduced pressure at 40 °C (almost until dryness) and subsequently purified using solid-phase extraction on a short glass column filled with the RP-C18 bed. Interfering compounds were flushed with 40 % methanol. The fraction containing saponins was eluted with 80 % methanol and then evaporated under reduced pressure until the total methanol was disposed of. The final extract was frozen at −50 °C and lyophilized using a Christ Gamma 2–16 LSC freeze-drier.

Samples of the fermentation fluid were collected under anaerobic conditions from each vessel 3 h before feeding time (at 8:00 am) for the last five days of each run. The fermentation fluid was analyzed for pH, volatile fatty acids (**VFA**), ammonia, and protozoa. The fermentation gas was collected at 11:00 am in a gas-tight collection bag (Tecobag 81; Tesseraux Container, Bürstadt, Germany) for the methane production measurement. The disappearance of DM was determined by analyzing feed residues in nylon bag samples taken before feeding for the last six days of each run.

2.1.2. In vivo experiment using rumen-cannulated dairy cows (Experiment 3)

Six multiparous lactating Polish Holstein-Friesian cows (612 ± 24 kg body weight; 5–6 months in lactation) fitted with rumen cannulas (2 C, 4 in., Bar Diamond, Parma, Idaho, USA) were used in a replicated 3 × 3 Latin square design with three 26-d periods. The three experimental diets were based on partial replacement of GS with KLS or VLS in the high-forage diet containing MS, as described earlier: MGS, MKLS, and MVLS (Table 1). Diets were offered as partial mixed rations (PMR) along with a top-dress concentrate supplement (Table 1). Cows were fed the PMR ad libitum (i.e., 5% orts as fed) and equal meals at 6 a.m. and 6 pm. The top-dress supplement was provided at a rate of 2 kg/d/cow when PMR was served. The DM concentration of silages were determined weekly and diets were adjusted when necessary. Cows were housed in individual tie-stalls, bedded with wood shavings as needed, with free water access. Cows were milked twice daily at 5:30 am and 5:30 pm.

During the sampling period (days 22–26), the amounts of fresh PMR offered and refused (orts) were recorded daily. Samples of orts for each cow and the fresh PMR for each treatment were collected throughout the sampling period and stored at −20 °C until submitted for DM and chemical analysis. On day 24 of each experimental period, starting at 6 a.m., total feces were collected over 24 h to determine total-tract digestibility coefficients. Subsamples (5%, w/w) of feces were stored at −20 °C for analysis for DM, OM, aNDFom, and CP. On day 26, a 600-mL sample of ruminal fluid was collected from three locations in the midventral sac of the rumen just before (0 h) and 3 h and 6 h after the 6 a.m. feed (Grummer et al., 1993). Ruminal fluid samples were strained through four layers of cheesecloth and the pH was immediately measured. One sample of filtered rumen fluid was stored at −20 °C for further analysis of FA composition. Another sample of filtered rumen fluid (3.6 mL) was preserved with 0.4 mL of 46 mM $HgCl_2$ solution and stored at −20 °C for later determination of VFA and ammonia-N. Subsequently, rumen fluid samples were strained through two layers of cheesecloth and the rumen fluid was fixed with 4% of formalin (6:1 ratio) for protozoa counting.

2.1.3. In vivo experiment using commercial dairy cows (Experiment 4)

Fifty-four multiparous lactating Polish Holstein-Friesian cows [600 ± 30.4 kg body weight, 2.4 ± 0.25 parity, 155 ± 29 d in milk, and 30 ± 2.1 kg/d milk production; (mean ± SD)] were used in a complete randomized design with 21 day adaptation periods and five-day sampling periods (a total of 26 days experimental period). Cows were randomly allocated to three dietary groups (eighteen cows in each group): MGS, MKLS, or MVLS. Diets were offered as a partial mixed ration (PMR) along with a separate feed of a concentrate supplement (Table 1). Cows were fed the PMR as equal meals at 6 a.m. and 6 pm. During the first eight days of each adaptation stage, the PMR was fed for *ad libitum* (i.e., 5% orts as fed). From day 9 onwards, cows were provided with restricted amounts

of PMR, which was 95 % of the average PMR intake of the previous 5 d. The concentrate supplement was fed at 2 kg/d/cow using a computer-controlled feeder station (De Laval FP 204, Tumba, Sweden). Dry matter intake was maintained at 24.0 ± 0.5 kg/d during the experiment. The DM concentration of silages were determined weekly and diets were adjusted when necessary. Cows were milked twice daily at 5:30 am and 5:30 pm.

Samples of fresh PMR for each treatment were collected throughout the sampling period (days 22–26) and stored at −20 °C until submitted for DM and chemical analysis. Daily milk yields were recorded throughout the sampling period using a milk meter (WB Ezi-Test Meter 33 kg; True-Test, Manukau, New Zealand). Milk samples were collected at each milking from all cows throughout the sampling period (days 22–26). Morning milk samples were stored at 4 °C until the evening samples were collected. The samples were subsequently pooled according to morning and evening milk yield and prepared in two equal parts: One aliquot was immediately analyzed for fat, CP, and lactose by infrared analysis (MilkoScan 255 A/S N, FossElectric, Hillerød, Denmark) and the rest was stored at −20 °C for FA analysis. Throughout days 22–26, methane production was continuously measured during the feeding of concentrate supplements at the computer-controlled feeder station (De Laval, type FP 204, Tumba, Sweden). The CH_4 concentration was measured using NDIR (nondispersive infrared spectroscopy) system operating in the near-infrared spectrum (detector 1210 Gfx Servomex 4100, Servomex, Crowborough, UK) (Sypniewski et al., 2019).

2.2. Sample analysis

2.2.1. Chemical composition of feeds

Thawed PMR samples were dried at 55 °C for 24 h, milled to pass through a 1-mm screen (Arthur H. Thomas, Philadelphia, PA, USA), and composited by treatment before chemical analysis. Samples were analyzed for analytical DM (method no. 934.01), ash (method no. 942.05), crude protein (**CP**; Kjel-Foss Automatic 16,210 analyzer, Foss Electric, Hillerød, Denmark; method no. 976.05), and ether extract [(**EE**; Soxhlet System HT analyzer; Foss Electric, Hillerød, Denmark; method no. 973.18); AOAC, 2007; Horwitz and Latimer, 2007]. Ash-free adjusted neutral detergent fiber (**aNDFom**) was determined using heat-stable α-amylase and sodium sulfite (Van Soest et al., 1991) in an Fibertech 1020 Analyzer (Foss., Analytical AB, Höganäs, Sweden) and reported on an ash-free basis.

2.2.2. Ruminal fermentation characteristics

The pH was immediately determined after sample collection using a pH meter (Elmetron CP-104, Zabrze, Poland). Ammonia concentration was determined using the colorimetric Nessler method and the VFA was analyzed as described by Szczechowiak et al. (2016). Briefly, preserved ruminal fluid samples were analyzed using a gas chromatograph (Varian CP 3380; Sugarland, TX, USA) equipped with an injector at 120 °C, a flame ionization detector at 230 °C, and a capillary column (30 m × 0.25 mm; Agilent HP-Innowax, 19091N-133, Agilent Technologies, Santa Clara, CA, USA). The VFA peaks were qualitatively and quantitatively identified using external standards prepared by mixing individual VFA purchased from Fluka (Sigma Aldrich, MO, USA). MS Work Station 5.0 was used to process the data.

2.2.3. In vitro digestible dry matter

In Experiments 1 and 2, the concentration of digestible DM was calculated as the amount of feed substrate (g/kg) that disappeared during in vitro incubation; this represented the difference in feed substrate DM weight before and after incubation.

2.2.4. Methane measurements

In Experiments 1 and 2, the methane concentration was measured using a gas chromatograph (SRI PeakSimple 310; Alltech, PA, USA) equipped with a thermal conductivity detector and a Carboxen 1000 column (mesh side 60/80, 15 FT × 1.8 INS.S; Supelco, Bellefonte, USA), in line with the protocol described by Szczechowiak et al. (2016). In exp. 3, methane production was estimated theoretically (fermentation balance for the measured molar proportion of VFA and the OM fermented in the rumen) (Wolin, 1960). In Experiment 4, methane production was continuously measured by infrared methane analyzers (Servomex 4000 Series, Servomex, Jarvis Brook, UK) and employing 1210 Gfx modules using the gas filter correlation technique. Two infrared methane analyzers were used with a range of 0–500 ppm (0–625 mg/m^3) for ambient air and 0–1000 ppm (0–1250 mg/m^3) for that feeding station through which the concentrates were supplied. Air samples were continuously collected from the feed bins in the feeder station at 15 L/min via an 8-mm diameter polyethylene tube (approximately 25 m in length) connected to a gas panel. The gas samples were distributed to the inlet port of the analyzer with a flow rate of 0.6 L/min. An additional part of the gas sample was driven by a by-pass line to decrease system response time. Air was drawn through the system by an external pump between the outlet of the feeding station and the gas inlet to the sample panel. Methane concentrations were logged at 2 s intervals on a computer via RS-232, using software with a database system (AnaGaz, Wrocław, Poland). Before each sampling stage, the analyzers were calibrated using standard calibration gases: nitrogen N5.0 and 1210 ppm CH_4 in N_2 (Multax, Zielonki-Parcela, Poland).

2.2.5. Ruminal microbial population

In Experiments 1, 2, and 3, protozoa counts were performed using a drop (100 μL) of buffered ruminal fluid under a light microscope (Zeiss, Primo Star no. 5, Jena, Germany) following Michalowski et al. (1986).

In Experiment 3, the methanogens and total bacteria were quantified by fluorescence in situ hybridization (FISH), following Józefiak et al. (2013) with some modifications. Briefly, 50 μL of ruminal fluid was diluted in phosphate-buffered saline (PBS) and pipetted onto 0.22 μm polycarbonate filters (Frisenette K02BP02500) and vacuumed (Vacuport Vacuum, KNF Neuberger). After vacuuming, the filters were transferred onto cellulose disks for dehydration in an ethanol series (50 %, 80 %, and 90 %; 3 min each).

For each sample, a series of identical filters was prepared to allow determination of optimal hybridization. Hybridizations were carried out in 50 µL of hybridization buffer (0.9 M NaCl; 20 mM Tris/HCl, pH 7.2; 0.01 % SDS) containing oligonucleotide probes [all methanogens (S-D-Arch-0915-a-A-20) and two order-specific probes: S-O-Mmic-1200-a-A-21 for *Methanomicrobiales* and S-F-Mbac-0310-a-A-22 for *Methanobacteriales*; Szczechowiak et al., 2016]. After hybridization, the filters were washed with washing buffer (20 mM Tris/HCl, pH 7.2; 0.01 % SDS; 5 mM EDTA) for 20 min at 48 °C. The filters were rinsed gently in distilled water, air-dried, and mounted on object glasses with VectaShield antifading agent (Vector Laboratories nr. H-1000) containing 4′,6-diamidino-2-phenylindole (DAPI). To distinguish the total count of bacteria (DAPI) from other microorganisms in the rumen fluid, filters were maintained at 4 °C for one hour in the dark until visualization using an Axio Imager M2 microscope (Carl Zeiss Iberia, Madrid, Spain).

2.2.6. FA analysis

Samples of fatty acid composition in ruminal fluid (Experiment 3), milk (Experiment 4), and dried ground feed (MGS, MKLS, and MVLS) were analyzed following to Szczechowiak et al. (2016). Briefly, 3-mL of 2 M NaOH was added to 2500 mg, 500 mg, and 100 mg of ruminal fluid, milk, and feed samples, respectively, for hydrolysis of the samples in a closed system using 15-mL screw-cap Teflon-stoppered Pyrex tubes. The hydrolyzed samples were incubated in a block heater at 90 °C for 40 min. Then, samples were extracted and esterified using 0.5 M NaOH in methanol and subsequently converted to FA methyl esters (**FAME**) using boron trifluoride (1.3 M; Fluka-Sigma Aldrich, St. Louis, MO, USA). A gas chromatograph (456-GC, Bruker, USA) equipped with a flame ionization detector and a 100-m fused-silica capillary column was used (0.25 mm i.d.; coated with 0.25 µm Agilent HP; Chrompack CP7420; Agilent Technologies, Santa Clara, CA, USA). Hydrogen at a flow rate of 1.3 mL/min was used as the carrier gas. The injector and detector temperatures were 200 °C and 250 °C, respectively. The oven temperature was programmed as follows: initially 120 °C for 7 min, then increased by 7 °C per min to 140 °C, where it was held for 10 min before being increased by 4 °C per min to 240 °C. A 1-µL sample was injected into the GC column. The peaks were identified by comparison with the retention times of appropriate FAME standards (37 FAME Mix, Sigma Aldrich, PA, USA). Moreover, the conjugated linoleic acid peaks were identified by comparison with the retention times of a reference standard (a mixture of *cis*- and *trans*- 911 and 1012-octadecadienoic acid methyl esters; Sigma Aldrich, PA, USA) using Galaxie Work Station 10.1 (Varian, CA, USA). Fatty acid compositions were expressed as g/100 g total FA.

2.3. Statistical analysis

In Experiments 1 and 2, the data from the Rusitec equipment (methane, pH, VFA, ammonia, digestible DM, and protozoa) were averaged by fermenter and analyzed using a model that included the fixed effect of treatment, the random effect of the block (incubation run), and the random effect of the fermenter within the treatment using PROC MIXED of SAS (SAS 9.2; SAS Institute, Cary, NC, USA). Sums of squares for the treatment effects were further separated into single degree of freedom comparisons to test for the significance of preplanned contrasts as follows: (1) partial replacement of GS by KLS (MGS vs. MKLS + sMKLS in Experiment 1) or VLS

Table 2
Ruminal fermentation characteristics measured in Rusitec fermenters as affected by partially replacing grass silage (GS) with Kometa lucerne silage (KLS) in the diet or saponin extract supplementation.

Item	Treatments [1]			SEM [2]	Contrast [3]	
	MGS	MKLS	sMKLS		G vs. L	SAP
TGP, mL/d	4558	4470	5082	102.1	0.21	0.008
Methane, mL/d	305	292	316	15.9	0.97	0.63
pH	6.93	6.85	6.81	0.014	<0.001	0.012
Total VFA, mmol/L	60.4	58.7	56.7	2.44	0.62	0.76
VFA proportion, mol/100 mol						
Acetate (A)	48.9	40.9	41.9	0.89	<0.001	0.51
Propionate (P)	22.9	22.9	24.1	1.11	0.82	0.68
Butyrate	16.1	18.6	17.2	0.65	0.20	0.38
Valerate	7.53	7.76	7.87	0.221	0.55	0.85
Isovalerate	3.14	3.56	2.74	0.189	0.97	0.086
Isobutyrate	1.35	5.75	6.03	0.493	<0.001	0.71
A:P ratio	2.17	1.65	1.59	0.074	<0.001	0.74
Ammonia, mmol/L	4.54	5.08	5.45	0.146	0.011	0.21
Digestible DM, g/kg	441	488	504	9.29	0.002	0.31
Protozoa						
Total, $\times 10^4$/mL	3.91	3.64	3.65	0.129	0.42	0.98
Entodiniomorpha, $\times 10^4$/mL	3.85	3.57	3.59	0.132	0.43	0.96
Holotricha, $\times 10^4$/mL	0.063	0.047	0.047	0.050	0.13	1.00

[1] MGS = a high-forage diet (65:35 forage:concentrate ratio; DM basis) containing maize silage (MS) and GS as the main forage sources; MKLS = a high-forage diet containing MS and KLS as the main forage sources; sMKLS = a high-forage diet containing MS and KLS as the main forage sources and supplemented with saponin extract powder.

[2] SEM = standard error of mean.

[3] Significance of effects due to partially replacing GS with KLS in the diet (G vs. L; MGS vs. MKLS + sMKLS) and supplementation of MKLS diet with saponin extract (SAP; MKLS vs. sMKLS).

(MGS vs. MVLS + sMVLS in Experiment 2) in the diet and (2) supplementation of the MKLS (MKLS vs. sMKLS in Experiment 1) or MVLS (MVLS vs. sMVLS in Experiment 2) diet with saponin extract. In Experiment 3, the intake and digestibility data were averaged by cow before analysis. Intake, digestibility, and rumen FA composition data were analyzed using a model that included treatment and period as the fixed effects, and square and cow within square as the random effects. Data on the ruminal fermentation characteristic (pH, VFA, ammonia, and microbes) obtained from the three sampling points (before and 3 h and 6 h after the 6:00 am feeding) were analyzed as repeated measures using the same model, with the addition of the fixed effects of sampling time (i.e., hour) and treatment × sampling time. Because there were no interactions between treatment and sampling time for the ruminal fermentation parameters, only averages over time are presented. In Experiment 4, the milk production performance, milk FA composition, and methane production data were average by cow before analysis, and were analyzed using a model that included the fixed effect of treatment and the random effect of the cow within the treatment using PROC MIXED of SAS. In both Experiments 3 and 4, the sums of squares for treatment effects were further separated into single degree of freedom comparisons to test for the significance of preplanned contrasts as follows: (1) partial replacement of GS with LS in the diet (MGS vs. MKLS + MVLS) and (2) cultivar type of ensiled lucerne in the diet (MKLS vs. MVLS). Least square means are reported, and treatment effects were taken as significant or as tending towards significance at $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively.

3. Results

3.1. In vitro experiments

The ruminal fermentation characteristics for the Rusitec fermenters in Experiment 1 are shown in Table 2. Replacing GS with KLS in a high-forage diet containing MS did not affect total gas production in Rusitec fermenters. However, the supplementation of saponin extract in the MKLS diet increased total gas production ($P < 0.01$). Methane production was not affected by replacing GS with KLS or by supplementing saponin extract in the MKLS diet. Both replacing GS with KLS and supplementing with saponin extract led to decreased rumen pH ($P \leq 0.01$). Replacing GS with KLS or supplementing with saponin extract did not affect total VFA concentration. The molar proportion of acetate decreased ($P < 0.01$), isobutyrate increased ($P < 0.01$), and propionate was not affected by the replacement of GS with KLS in the diet. The supplementation of MKLS diet with saponin extract tended ($P = 0.09$) to decrease the molar proportion of isovalerate, but did not affect acetate or propionate values. Ammonia concentration was increased ($P = 0.01$) by replacing GS with KLS in the diet but was not affected by supplementation with saponin extract. DM disappearance was increased ($P < 0.01$) when GS was replaced with KLS, but the corresponding value was not affected by the addition of saponin extract. The numbers of *entodiniomorpha* and *holotricha* were not affected by replacing GS with KLS or by the presence of saponin.

The ruminal fermentation characteristics for the Rusitec fermenters in Experiment 2 are shown in Table 3. Total gas or methane production were not affected by the replacement of GS by VLS in a high-forage diet containing MS or by supplementing the MVLS diet with saponin extract. Ruminal pH decreased when GS was replaced by VLS ($P < 0.01$) but was not affected by supplementation with

Table 3
Ruminal fermentation characteristics measured in Rusitec fermenters as affected by partially replacing grass silage (GS) with Verko lucerne silage (VLS) in the diet or saponin extract supplementation.

Item	Treatments [1]			SEM [2]	Contrast [3]	
	MGS	MVLS	sMVLS		G vs. L	SAP
TGP, mL/d	5046	4983	5111	111.6	0.99	0.68
Methane, mmol/d	340	336	335	21.4	0.92	0.99
pH	6.94	6.85	6.84	0.014	<0.001	0.86
Total VFA, mmol/L	58.5	56.5	60.9	1.61	0.95	0.29
VFA proportion, mol/100 mol						
Acetate (A)	50.5	48.1	44.8	0.87	0.018	0.099
Propionate (P)	22.6	22.8	24.0	0.82	0.67	0.54
Butyrate	15.8	17.4	15.7	0.71	0.64	0.33
Valerate	6.76	7.11	7.33	0.207	0.31	0.66
Isovalerate	2.95	2.56	3.22	0.178	0.87	0.14
Isobutyrate	1.31	2.14	4.98	0.374	<0.001	<0.001
A:P ratio	2.29	2.27	1.94	0.108	0.39	0.22
Ammonia, mmol/L	4.58	6.20	6.25	0.225	<0.001	0.83
Digestible DM, g/kg	437	469	477	10.2	0.10	0.77
Protozoa						
Total, × 10^4/mL	3.71	3.83	4.07	0.133	0.43	0.51
Entodiniomorpha, × 10^4/mL	3.64	3.76	3.99	0.132	0.42	0.52
Holotricha, × 10^4/mL	0.074	0.067	0.083	0.049	0.91	0.21

[1] MGS = a high-forage diet (65:35 forage:concentrate ratio; DM basis) containing maize silage (MS) and GS as the main forage sources; MVLS = a high-forage diet containing MS and VLS as the main forage sources; sMVLS = a high-forage diet containing MS and VLS as the main forage sources and supplemented with saponin extract powder.

[2] SEM = standard error of mean.

[3] Significance of effects due to partially replacing GS with VLS in the diet (G vs. L; MGS vs. MVLS + sMVLS) and supplementation of MVLS diet with saponin extract (SAP; VKLS vs. sVKLS).

saponin extract. Replacing GS with VLS or supplementing with saponin extract did not affect total VFA concentration. The molar proportion of acetate decreased ($P = 0.02$), isobutyrate increased ($P < 0.01$), and propionate was not affected by the replacement of GS by VLS.

Similarly, the molar proportion of acetate tended to decrease ($P = 0.10$), isobutyrate increased ($P < 0.01$), and propionate was not affected when saponin extract was added. The concentration of ammonia increased ($P = 0.01$) when GS was replacing by VLS, but was not affected by the addition of saponin extract. DM disappearance tended ($P = 0.10$) to increase when GS was replaced by VLS, but was not affected by supplementation with saponin extract. The numbers of *entodiniomorpha* and *holotricha* were not affected when GS was replaced by VLS or by the saponin extract treatment.

3.2. *In vivo experiments*

The ruminal fermentation characteristics in the rumen-cannulated cows in Experiment 3 are shown in Table 4. Ruminal pH declined ($P < 0.001$) when GS was replaced by LS in the high-forage diet containing MS, where the response tended ($P = 0.08$) to be greater for the MVLS diet than for the MKLS diet. Replacing GS by LS led to a decrease in total VFA concentration ($P < 0.001$), but no differences attributed to lucerne cultivar were observed in the cows. We observed decreases in the molar proportions of acetate and isobutyrate ($P < 0.001$) and increases in propionate, butyrate, and valerate ($P \leq 0.04$) when GS was replaced by LS. For the MVLS diet, the molar proportion of acetate tended to be larger ($P = 0.07$), that of propionate was smaller ($P = 0.07$), and that of isobutyrate was smaller ($P = 0.05$) than in the case of the MKLS diet. Replacing GS by LS led to increased ammonia concentration ($P < 0.001$), but no differences attributed to lucerne cultivar were observed in the cows. Replacing GS by LS decreased the total bacteria, total protozoa (including *entodiniomorpha* and *holotricha*), and the total methanogens ($P \leq 0.03$), with the responses of cows, fed the MVLS diet being were greater ($P \leq 0.009$) than those of the cows fed the MKLS diet. Replacing GS by LS led to an increase in DM intake ($P < 0.001$), and the cows fed the MVLS diet had a greater intake than the cows fed MKLS diet. Replacing GS by LS tended to increase total-tract digestibility of CP ($P = 0.09$); but did not affect DM, OM, or aNDFom digestibility; no differences were observed between lucerne cultivar.

Ruminal FA composition in the rumen-cannulated cows in Experiment 3 are shown in Table 5. Replacing GS by LS in the diet

Table 4
Ruminal fermentation characteristics measured in rumen-cannulated cows as affected by partially replacing grass silage (GS) with Kometa (KLS) or Verko lucerne silage (VLS) in the diet.

Item	Treatments [1]			SEM [2]	Contrast [3]	
	MGS	MKLS	MVLS		G vs. L	CUL
pH	6.22	6.13	6.06	0.018	<0.001	0.079
Total VFA, mmol	117	105	107	0.88	<0.001	0.26
VFA proportion, mol/100 mol						
Acetate (A)	62.0	59.3	60.2	0.21	<0.001	0.068
Propionate (P)	23.1	24.7	23.9	0.15	<0.001	0.069
Butyrate	11.7	12.3	12.1	0.10	0.042	0.53
Valerate	1.55	1.73	1.74	0.022	<0.001	0.93
Isovalerate	1.08	1.14	1.09	0.014	0.25	0.21
Isobutyrate	0.79	0.71	0.68	0.007	<0.001	0.050
A:P ratio	2.72	2.41	2.54	0.024	<0.001	0.016
Ammonia, mmol	8.27	9.77	9.67	0.184	<0.001	0.80
Methane [4]	0.62	0.53	0.56	0.004	<0.001	0.022
Total bacteria, $\times 10^8$/mL	163	138	102	2.27	<0.001	<0.001
Total protozoa, $\times 10^5$/mL	20.7	17.5	15.7	0.26	<0.001	<0.001
Holotricha, $\times 10^5$/mL	6.62	5.60	4.52	0.165	<0.001	0.004
Entodiniomorpha, $\times 10^5$/mL	14.4	12.4	11.1	0.19	<0.001	0.001
Total methanogens, $\times 10^8$/mL	5.93	5.78	5.18	0.094	0.023	0.009
Methanobacteriales, $\times 10^7$/mL	4.72	4.49	4.16	0.086	0.034	0.12
Methanomicrobiales, $\times 10^7$/mL	3.96	3.66	3.34	0.072	0.002	0.068
DMI [5]	23.2	23.7	24.2	0.09	<0.001	0.009
Total-tract digestibility [6], g/kg DM						
DM	624	624	620	1.83	0.70	0.47
OM	639	641	636	1.81	0.92	0.28
aNDFom	522	520	521	1.87	0.72	0.90
CP	658	681	675	5.43	0.094	0.66

[1] MGS = a high-forage diet (65:35 forage:concentrate ratio; DM basis) containing maize silage (MS) and GS as the main forage sources; MKLS = a high-forage diet containing MS and KLS as the main forage sources; MVLS = a high-forage diet containing MS and VLS as the main forage sources.
[2] SEM = standard error of mean.
[3] Significance of effects due to partially replacing GS with KLS or VLS in the diet (G vs. L; MGS vs. MKLS + MVLS) and cultivar type of ensiled lucerne in the diet (CUL; MKLS vs. MVLS).
[4] Methane (CH_4) estimation (Wolin, 1960).
[5] DMI = dry matter intake.
[6] DM, dry matter; OM, organic matter; aNDFom, ash free adjusted neutral detergent fiber; CP, crude protein.

Table 5
Ruminal fatty acid composition (g/100 g of total FA) in rumen-cannulated cows as affected by partially replacing grass silage (GS) with Kometa (KLS) or Verko lucerne silage (VLS) in the diet.

Item	Treatments [1]			SEM [2]	Contrast [3]	
	MGS	MKLS	MVLS		G vs. L	CUL
C8:0	0.09	0.11	0.13	0.007	0.017	0.27
C10:0	0.09	0.15	0.15	0.008	0.003	0.80
C12:0	0.28	0.33	0.41	0.009	<0.001	<0.001
C14:0	0.78	0.79	0.98	0.022	0.026	0.001
C14:1	0.95	1.12	1.04	0.011	<0.001	0.003
C16:0	16.9	17.4	17.3	0.09	0.032	0.83
C16:1 *cis*-9	0.27	0.22	0.42	0.017	0.15	<0.001
C18:0	45.2	40.1	42.3	0.43	<0.001	0.032
C18:1 *cis*-9	5.26	5.36	5.38	0.091	0.57	0.94
C18:1 *trans*-10	0.92	0.97	1.04	0.023	0.092	0.26
C18:1 *trans*-11	3.99	3.81	3.46	0.042	<0.001	<0.001
C18:2 *cis*-9, *cis*-12	4.90	5.99	5.37	0.093	<0.001	0.006
C18:2 *cis*-9, *trans*-11	0.94	0.97	0.61	0.026	0.006	<0.001
C18:2 *trans*-10, *cis*-12	0.24	0.27	0.44	0.011	<0.001	<0.001
C18:3 *cis*-9, *cis*-12, *cis*-15	1.09	1.02	1.15	0.020	0.80	0.008
C20:3n-6	0.53	0.52	0.43	0.011	0.015	<0.001
C20:4n-6	0.18	0.22	0.31	0.013	0.004	0.005
C20:5n-3	0.23	0.35	0.20	0.012	0.043	<0.001
C22:6n-3	0.13	0.14	0.19	0.009	0.069	0.017
C24:0	0.43	0.52	0.59	0.022	0.008	0.21
Total SFA [4]	66.4	62.3	66.6	0.332	0.007	<0.001
Total MUFA [5]	23.5	26.6	24.0	0.221	<0.001	<0.001
Total PUFA [6]	10.1	11.1	9.40	0.157	<0.001	<0.001

[1] MGS = a high-forage diet (65:35 forage:concentrate ratio; DM basis) containing maize silage (MS) and GS as the main forage sources; MKLS = a high-forage diet containing MS and KLS as the main forage sources; MVLS = a high-forage diet containing MS and VLS as the main forage sources.
[2] SEM = standard error of mean.
[3] Significance of effects due to partially replacing GS with KLS or VLS in the diet (G vs. L; MGS vs. MKLS + MVLS) and cultivar type of ensiled lucerne in the diet (CUL; MKLS vs. MVLS).
[4] SFA = saturated fatty acids.
[5] MUFA = monounsaturated fatty acids.
[6] PUFA = polyunsaturated fatty acids.

increased ruminal concentrations of C8:0, C10:0, C12:0, C14:0, and C16:0 ($P \leq 0.03$), while the responses of C12:0 and C14:0 were greater for cows fed the MVLS diet than for those fed the MKLS diet. The concentration of C18:0 in the rumen decreased when GS was replaced by LS in the diet ($P < 0.001$), with the response of cows fed the MKLS diet being greater than that of cows fed the MKLS diet

Table 6
Milk production performance of commercial dairy cows as affected by partially replacing grass silage (GS) with Kometa (KLS) or Verko (VLS) lucerne silage in the diet.

Item	Treatments [1]			SEM [2]	Contrast [3]	
	MGS	MKLS	MVLS		G vs. L	CUL
Milk yield						
Milk, kg/d	27.8	28.1	28.2	0.14	0.21	0.72
3.5 % FCM [4], kg/d	30.3	31.3	29.5	0.22	0.77	0.001
ECM [5], kg/d	28.4	29.5	27.8	0.19	0.55	<0.001
Fat, g/d	1127	1206	1057	12.7	0.85	<0.001
Protein, g/d	930	946	962	4.75	0.019	0.18
Lactose, g/d	1386	1436	1372	8.64	0.32	0.002
Milk composition						
Fat, g/kg	40.1	41.9	38.9	0.45	0.69	0.007
Protein, g/kg	33.9	33.9	34.3	0.09	0.21	0.039
Lactose, g/kg	49.7	51.7	49.4	0.14	<0.001	<0.001
Methane, ppm	317	277	290	3.42	<0.001	0.002

[1] MGS = a high-forage diet (65:35 forage:concentrate ratio; DM basis) containing maize silage (MS) and GS as the main forage sources; MKLS = a high-forage diet containing MS and KLS as the main forage sources; MVLS = a high-forage diet containing MS and VLS as the main forage sources.
[2] SEM = standard error of mean.
[3] Significance of effects due to partially replacing GS with KLS or VLS in the diet (G vs. L; MGS vs. MKLS + MVLS) and cultivar type of ensiled lucerne in the diet (CUL; MKLS vs. MVLS).
[4] 3.5 % fat-corrected milk.
[5] Energy corrected milk.

($P = 0.03$). Replacing GS by LS in the diet increased C14:1 ($P < 0.001$) but did not affect C16:1 concentrations in the rumen. We observed greater concentrations of ruminal C14:1 ($P = 0.003$) and lower concentrations of C16:1 ($P < 0.001$) in cows fed the MKLS diet than in cows fed the MVLS diet. Replacing GS by LS in the diet had no effect on C18:1 *cis*-9, tended to increase C18:1 *trans*-10 ($P = 0.09$), and decreased C18:1 *trans*-11 ($P < 0.001$) in the rumen; the response of C18:1 *trans*-11 was greater in cows fed the MVLS diet ($P < 0.001$). Replacing GS by LS in the diet increased the ruminal concentrations of C18:2 *cis*-9, *cis*-12 and C18:2 *trans*-10, *cis*-12 ($P < 0.001$); the cows fed the MKLS diet had greater C18:2 *cis*-9, *cis*-12 ($P = 0.006$) and lower C18:2 *trans*-10, *cis*-12 ($P < 0.001$) than the cows fed the MVLS diet. The concentrations of C18:2 *cis*-9, *trans*-11 decreased when GS was replaced by LS in the diet ($P < 0.001$), while the response observed was due to the MVLS diet ($P < 0.001$). Replacing GS by LS in the diet did not affect ruminal concentrations of C18:3 *cis*-9, *cis*-12, or *cis*-15, but cows fed the MVLS diet showed greater C18:3 *cis*-9, *cis*-12, *cis*-15 in the rumen than those fed the MKLS diet ($P = 0.008$). Replacing GS by LS decreased the concentration of total saturated FA in the rumen ($P = 0.007$); this response was due to using the MKLS diet instead of the MVLS diet ($P < 0.001$). Monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) concentrations in the rumen increased ($P < 0.001$) when GS was replaced by LS; this response was mainly due to using the MKLS diet instead of the MVLS diet ($P < 0.001$).

Milk production performance and methane production in the lactating dairy cows in Experiment 4 are shown in Table 6. Replacing GS by LS in the high-forage diet containing MS increased milk protein yield and milk lactose content ($P \leq 0.02$) but did not affect milk yield, milk fat content, milk fat yield, milk protein content, or milk lactose yield. The MVLS diet resulted in lower milk fat content and yield and milk lactose content and yield ($P \leq 0.007$), and higher milk protein content ($P = 0.04$) than in the MKLS diet; there was no effect on milk yield. Methane production decreased when GS was replaced by LS ($P < 0.001$) and the response to the MKLS diet was greater than the response to the MVLS diet ($P = 0.002$).

The milk FA composition in the lactating dairy cows in Experiment 4 is shown in Table 7. The milk fat concentrations of C8:0, C10:0, C12:0, C14:0, and C16:0 increased when GS was replaced by LS in the diet ($P < 0.001$), and the responses of C10:0 and C14:0 were greater for cows fed the MVLS diet than for those fed the MKLS diet ($P \leq 0.009$). Replacing GS by LS in the diet led to a decrease in the C18:0 content of milk ($P = 0.046$), where the response was due to the use of the MKLS diet ($P < 0.001$). The milk fat content of C14:1 and C16:1 increased ($P \leq 0.002$) when GS was replaced with LS; the C14:1 response was greater for the MKLS diet ($P = 0.011$). Replacing GS by LS decreased the concentrations of C18:1 *cis*-9, C18:1 *trans*-10, and C18:1 *trans*-11 in milk ($P < 0.001$), while the responses of C18:1 *cis*-9 and C18:1 *trans*-10 were greater ($P < 0.001$) and the response of C18:1 *trans*-11 was lower ($P = 0.004$) in cows

Table 7
Milk fatty acid composition (g/100 g of total FA) of commercial dairy cows as affected by partially replacing grass silage (GS) with Kometa (KLS) or Verko (VLS) lucerne silage in the diet.

Item	Treatments [1]			SEM [2]	Contrast [3]	
	MGS	MKLS	MVLS		G vs. L	CUL
C8:0	0.94	1.07	1.08	0.011	<0.001	0.80
C10:0	2.24	2.62	2.78	0.029	<0.001	0.009
C12:0	2.63	3.27	3.37	0.036	<0.001	0.17
C14:0	9.01	10.3	11.1	0.09	<0.001	<0.001
C14:1	0.91	1.04	0.96	0.013	<0.001	0.011
C16:0	27.5	28.3	29.4	0.16	<0.001	0.001
C16:1 *cis*-9	1.23	1.32	1.36	0.017	0.002	0.24
C18:0	11.1	9.76	11.5	0.11	0.046	<0.001
C18:1 *cis*-9	24.8	18.4	22.4	0.30	<0.001	<0.001
C18:1 *trans*-10	0.66	0.45	0.61	0.019	<0.001	<0.001
C18:1 *trans*-11	1.44	1.29	1.13	0.024	<0.001	0.004
C18:2 *cis*-9, *cis*-12	3.16	2.27	2.21	0.047	<0.001	0.54
C18:2 *cis*-9, *trans*-11	0.22	0.25	0.19	0.005	0.70	<0.001
C18:2 *trans*-10, *cis*-12	0.22	0.19	0.15	0.005	<0.001	<0.001
C18:3 *cis*-9, *cis*-12, *cis*-15	0.94	0.64	0.58	0.016	<0.001	0.045
C20:3n-6	0.34	0.32	0.12	0.009	<0.001	<0.001
C20:4n-6	0.07	0.09	0.07	0.002	0.042	<0.001
C20:5n-3	0.19	0.41	0.13	0.016	0.006	<0.001
C22:5n-3	0.19	0.34	0.14	0.009	0.003	<0.001
C22:6n-3	0.16	0.21	0.24	0.011	0.086	<0.001
C24:0	0.10	0.21	0.11	0.007	<0.001	<0.001
Total SFA [4]	57.8	61.4	64.6	0.28	<0.001	<0.001
Total MUFA [5]	36.3	32.9	31.3	0.22	<0.001	<0.001
Total PUFA [6]	5.78	5.63	3.94	0.087	<0.001	<0.001

[1] MGS = a high-forage diet (65:35 forage:concentrate ratio; DM basis) containing maize silage (MS) and GS as the main forage sources; MKLS = a high-forage diet containing MS and KLS as the main forage sources; MVLS = a high-forage diet containing MS and VLS as the main forage sources.
[2] SEM = standard error of mean.
[3] Significance of effects due to partially replacing GS with KLS or VLS in the diet (G vs. L; MGS vs. MKLS + MVLS) and cultivar type of ensiled lucerne in the diet (CUL; MKLS vs. MVLS).
[4] SFA = saturated fatty acids.
[5] MUFA = monounsaturated fatty acids.
[6] PUFA = polyunsaturated fatty acids.

fed the MVLS diet than in cows fed the MKLS diet. Replacing GS by LS in the diet decreased milk fat concentrations of C18:2 *cis*-9, *cis*-12 and C18:2 *trans*-10, *cis*-12 ($P < 0.001$), with the response of C18:2 *trans*-10, *cis*-12 being greater for the MVLS diet ($P < 0.001$). The milk fat concentration of C18:3 *cis*-9, *cis*-12, *cis*-15 decreased when GS was replaced by LS in the diet ($P < 0.001$) and the response was greater for cows fed the MVLS diet ($P = 0.045$). Replacing GS by LS in the diet increased the concentration of total saturated FAs in the milk ($P < 0.001$) and the response was lower for the MKLS diet ($P < 0.001$). Replacing GS by LS led to decreased milk fat concentrations of total MUFA and PUFA ($P < 0.001$), where the responses were lower for cows fed the MVLS diet ($P < 0.001$).

4. Discussion

Lucerne represents a rich source of secondary metabolites with bioactive properties, including saponins, which are important bioactive compounds whose effects on ruminal fermentation and methanogenesis are dependent on chemical structure, plant species, location in the plant, and the presence of other bioactive compounds in the diet (Bodas et al., 2012; Zhan et al., 2017; Kozłowska et al., 2020). Both in vitro and in vivo experiments were carried out.

4.1. In vitro experiments

The main assumption was that lucerne, as a source of bioactive compounds, could modulate ruminal fermentation and methane concentration. The effects of phytogenic additives, rich in secondary plant metabolites, on methane and ammonia production have been discussed in several studies (Cieslak et al., 2013). Some data have pointed to the antimicrobial properties of secondary plant metabolites against a wide range of rumen microorganisms, affecting rumen microbial fermentation and decreasing ammonia production (Coblentz and Grabber, 2013); other data suggest that there is no effect on ammonia concentration (Busquet et al., 2006). The effects of active components depend on their chemical structure, constituents, and concentration (Castillejos et al., 2006; Maulfair and Heinrichs, 2013). The types of saponin present in lucerne could be responsible for the range of different responses. The presence in the sapogenins of different substituents such as hydroxyl, hydroxymethyl, carboxyl, and acyl groups as well the differences in the composition, linkage, and number of sugar chains, accounts for significant structural variation, and thus variation in bioactivity (Patra and Saxena, 2009; Sidhu and Wadhwa, 2019). In Experiment 1, the assumption was confirmed for the decreases in the pH level and methane concentration and for the increases in DDM with MKLS and sMKLS. The increase in DDM in the groups with the lucerne additive was associated with a decrease in fiber and an increase in crude protein, resulting in an elevation of total degradable DM fractions (Chen et al., 2019). The research of Belanche et al. (2016a), who used ivy fruit saponins, also showed a correlation between methane emission reduction, strong antiprotozoal effects, and NDF reduction. In Experiment 2, no statistically significant effects were noted for methane or DDM with either MVLS or sMVLS. However, the experimental groups were numerically consistent with the results in Experiment 1. MVLS and sMVLS reduced methane concentration and increased DDM. Fllya et al. (2007) did note a pH level that was lower by 0.75 in their lucerne silage group than in their control group. Muck et al. (2007) cited an increased DDM associated with a decrease in the pH level for the lucerne silage treatments. Other studies also found lower acetic:propionic ratios, which may be associated with lower NDFs in the diet (Bodas et al., 2012). This observation was similar to our results, where MKLS, sMKLS, and numerically sMVLS decreased the acetic:propionic ratio in both experiments. However, it was only in the experiment with the Kometa lucerne silage that the result was statistically significant. Changes in VFA concentrations in Experiments 1 and 2 such as the decrease in the consumption of acetic acid and the increase in propionic and butyric acids could be due to the shift in hydrogen from the methane pathway, making it available to produce propionate (Norrapoke et al., 2014). Moreover, except for MVLS, all the treatments showed increased concentrations of isobutyrate, which probably arose from deamination of valine in the rumen ($P < 0.001$). These results are similar to those from studies of tea saponins, except for the concentration of isobutyrate, which was lower for supplementation with tea saponins than for the control group (Wang et al., 2019). Ammonia nitrogen did increase significantly compared to the control in both experiments with the lucerne silage-based diet, which agrees with the observations of Manatbay et al. (2014) of an increase in nitrogen ammonia when lucerne silage was used, resulting in a lower pH followed by the inhibition of absorption and the incorporation of ammonia into the microbial mass. The greater ammonia nitrogen concentrations in both experimental groups might partly result from a mismatch of energy and ammonia availability, and hence lower ammonia utilization. Under the same conditions, an excess of ruminal protein supply in lucerne silages might lead to N pollution, with more ammonia and nitrous oxide emissions (Chen et al., 2019). These results, in addition to the increase in the protozoa population with MVLS and sMVLS, are consistent with the results obtained in a previous in vitro experiment with lucerne silage using Hohenheim gas tests (Kozłowska et al., 2020). In this study, much lower microorganism populations were observed in the control and experimental in vitro groups than in the in vivo experiment. The results of the in vitro experiment were consistent with those of Kozłowska et al. (2020) using Hohenheim gas tests. The cause of this might be the different conditions in the RUSITEC fermenters and in cow rumens, which could negatively affect some microbial populations (Martínez et al., 2010). One possible reason for decreased protozoa population in fermenters during the incubation period is the exposure of the fermenter contents to atmospheric oxygen and the wash out of slow-growing microorganism from the fermenters (Moumen et al., 2009). For this reason, statistically significant changes might not have been observed in the microorganism population in vitro.

4.2. In vivo experiments

In Experiment 3 using cannulated cows, MKLS and MVLS had similar effects as in the in vitro studies, with lower pH values, lower acetate:propionate ratios, lower methane concentrations, and a higher ammonia concentration. The lower pH value favored the

population of propionate-producing bacteria, inhibiting the population of other microorganisms, dropping the acetate:propionate ratio (Molina-Botero et al., 2019). Lower acetate and isobutyrate concentrations were also observed. In the study of Aboagye et al. (2019) which looked at diets with the addition of tannins and gallic acid VFA concentration was higher than in control group, where lucerne silage without additions was employed. In the experiment where tea saponins were added, a decrease in total VFA was seen compared to the control diets to which tea saponins were not added (Guyader et al., 2017). Both of these studies had results similar to those of this experiment, but these effects were associated with negative changes in microbial population numbers, which were not observed in the in vitro studies.

Saponins have a negative impact on rumen protozoa number (Molina-Botero et al., 2019; Kozłowska et al., 2020), a fact which our findings support, given the decrease we observed in the number of *Holotricha* spp., *Entodiniomorpha* spp., and total protozoa count. This protozoal decrease is thought to result from the change in the protozoal cell membrane permeability of plant secondary metabolites and the formation of complexes with cholesterol in the protozoal cell membrane, resulting in cell lysis (Jayanegara et al., 2014). Hydrophobic interactions between triterpenoid saponins with such membrane cholesterol seem to be less effective in causing protozoa cell lysis than those with steroid saponins, which more often occur in the aerial parts of the lucerne used in those experiments (Jayanegara et al., 2014). Ruminal methanogenesis also decreased, which could relate to a decrease in the number of protozoa (Cieslak et al., 2013). It has been observed in previous studies that *Holotricha* protozoa seem to be key players in rumen methanogenesis (Belanche et al., 2016b). In the present study, it seems that the decrease in methanogens was associated with the decrease in the protozoa population. This was also confirmed by previous in vitro studies (Kozłowska et al., 2020). Ruminal methane production was reduced with the presence of plant secondary metabolites. This, as suggested by Kara et al. (2016), is associated with a decrease in the number of rumen microbes. Most species of rumen protozoa contain hydrogenosomes, a type of membrane-bound organelle producing hydrogen through malate oxidization. These protozoa can thus attract methanogens as endosymbionts. The symbiosis displayed by these protozoa-associated methanogens (PAM) is likely to be beneficial for both parties, as the hydrogen generated by the rumen protozoa could be utilized by the PAM in common (Jafari et al., 2017; Patra et al., 2017). About 16 % of rumen protozoa have been shown to contain methanogens, and PAMs are estimated to generate as much as 37 % of the total rumen methane (Jafari et al., 2017; Matthews et al., 2019). This study has confirmed that the use of a diet with the addition of lucerne silage in dairy cows can reduce methane emissions, possibly by reducing the rumen PAM population. The effect of saponins on rumen methane content is dependent on both their concentration and their source (Jayanegara et al., 2014). The treatment of dairy cows with tea saponins led to an increase in methane emission by as much as 14 % over the control group. Given the promising effects of tea saponins observed in the in vitro study (though not confirmed by the in vivo experiment) studies with both types of experiments once again show the importance of comprehensive research in developing new methane emission mitigating strategies (Guyader et al., 2017).

The decrease in methane production resulting from the MKLS and MVLS treatments may be due to the reduction of hydrogen, which acts as a substrate for rumen microbiota (Wencelová et al., 2015). The studies of Patra and Yu (2013, 2015) suggested that binary and ternary combinations of antimethanogenic inhibitors with complementary mechanisms of actions on methanogenesis may alter archaeal communities and decrease methane production additively, without negatively affecting ruminal fermentation or degradability. A greater concentration of ammonia is consistent with the levels of protein in the diets. Lucerne has high crude protein and soluble protein contents, which could lead to elevated ruminal ammonia concentrations and the excretion of excess N into the environment (Aboagye et al., 2019). As both lucerne diets have greater amounts of protein, and thus greater amounts of amino acids, it could be expected that they would contain more than diets based on grain or oilseed byproducts, major fiber sources, or low starch/high-NDF diets (Brooks et al., 2014); this is in line with the increase observed in ammonia. Saponin treatment resulted in a decrease in the protozoal population's utilization of ammonia to synthesize proteins, hence the observed increase in NH_3. Moreover, changes in ammonia concentration could also be explained by the levels of saponin in the experimental treatment being too low to affect the activity of certain strains of proteolytic bacteria. This was the case with treatment with another secondary metabolite, tannin, in the study of Szczechowiak et al. (2016).

Despite having no effect on milk productivity, the MVLS in Experiment 4 seem to have the ability to lower fat content and fat yield, as well as energy content all of which are associated with lower fat content. In the experiment of Guyarder et al. (2017), tea saponins reduced fat yield, lactose yield, and crude protein yield, as was also the case for saponins in this study. The literature suggests that inconsistency in forage components can leave diets vulnerable to reduced NDF content which, if insufficient, can result in reduced chewing activity and less saliva production, causing lower ruminal pH, thus altering ruminal fermentation and increasing the risk of milk fat depression (Mertens, 1997). It has been shown that the use of certain feed additives also alters ruminal lipid metabolism, resulting in the formation of specific biohydrogenation intermediates that directly inhibit milk fat synthesis. The conjugated isomer of linoleic acid (C18:2 *trans*-10, *cis*-12) has been shown to decrease milk fat (Pi et al., 2019). As expected, MKLS and MVLS had positive effects on the levels of C18: 2 *cis*-9 *cis*-12 and C18:2 *trans*-10 *cis*-12 in the rumen fluid (Table 5), as biohydrogenation leads to the production of conjugated linoleic acids isomers as intermediates (Szczechowiak et al., 2016). The proportion of C18:2 *cis*-9, *trans*-11 increased significantly only with the MKLS treatment. Despite the more promising fatty acid composition of the Kometa and Verko lucerne feed material used in the experimental groups, the changes in rumen fluid FA were not as significant as expected. This may be because grass silage was only partially substituted by Kometa and Verko lucerne silage in the experimental diets.

Few experiments have reported on the effects of lucerne silage on milk FA profile. This study suggests that the milk fat of dairy cows fed either MKLS or MVLS contained more C14:0 and C16:0, and less C18:1 *cis*-9, C18:2 *cis*-9 *cis*-12, and C18:3 *cis*-9 *cis*-12 *cis*-15, than that of cows fed a grass silage-based diet (Timmen and Patton, 1988; Kelly et al., 1998; Dhiman et al., 1999). Overall, the levels of SFAs were higher and the levels of MUFAs and PUFAs were lower for MKLS and MVLS than for GS. This may be due to the transformation of fatty acids in the rumen and mammary gland. In dairy cows, C14:0 derives mainly from de novo synthesis in the mammary glands (Kennelly and Glimm, 1998), whereas C16:0 may derive from either dietary consumption or de novo synthesis in the mammary gland

(Grummer et al., 1993). For both C14:0 and C16:0, de novo synthesis can be inhibited by long chain C18 UFA, like linoleic and linolenic acid (Palmquist et al., 1993; Beaulieu and Palmquist, 1995). In the present study, the higher proportion of C14:0 in the milk fat of cows fed MKLS and MVLS than in cows in the control group may be due to high de novo C14:0 synthesis, since the dairy cows fed more lucerne silage consumed less medium chain fatty acid than did the cows fed the grass silage-based diet. The C18:0 was higher ($P < 0.001$) in the MVLS treatment than in the cows fed MKLS (Table 7). A plausible explanation for this finding is that C18:0 is a major end-product of ruminal biohydrogenation of the C18 UFA (Loor et al., 2002), which was higher with MVLS than with MKLS and GS. An increase in C18:0 concentration is consistent with the studies of Henke et al. (2017) and arises from the mobilization of the body caused by a slightly negative energy balance. Studies on the effect of tannin extract have shown a reduction in C18:2 *cis*-9 *trans*-11 in milk compared with the control, which is consistent with these observations in the MVLS group. Furthermore, research with tannin extracts, another secondary metabolite, have shown the reduction of C18:2 *cis*-9 *trans*-11 in the milk compared with the control, which is consistent with our MVLS results (Henke et al., 2017). The rumenic acid in milk fat comes mainly from Δ9-desaturation of vaccenic acid in the mammary gland (Cappucci et al., 2018). Moreover, rumen biohydrogenation of vaccenic acid to C18:0 limits the availability of PUFA in ruminant products, such as meat and milk (Jafari et al., 2018). One explanation for the differences in FA proportion might be that the FA proportions in the rumen fluid are not identical to those in the duodenum, which is the main absorption site for FA into the blood (Szczechowiak et al., 2016). Moreover, this lack of consistency between FA in the rumen fluid and in milk may be due to the rumen passage rate, interaction with the basal diet, the varying amount of lipids in the diet, and the specific composition of secondary metabolites (Cappucci et al., 2018). Changes in the FA profile may be directly related to changes in the microbial population involved in the biohydrogenation process, as has been indicated in several studies (Szczechowiak et al., 2016; Bryszak et al., 2019). It has been shown that higher concentrations of some UFA in the rumen are not directly associated with the proportions of these FA found in milk. An example is C18: 2 *cis*-9 *cis*-12, which increased by 22 % in the rumen fluid but decreased in the milk concentrations. Biohydrogenation was more effective in the MKLS and MVLS groups, and fewer unsaturated acids were absorbed from the distal parts of the gastrointestinal tract into the blood. There was thus less UFA and less C18:2 *trans*-10 *cis*-12 in the milk samples, while in the rumen fluid, these fatty acids were found in higher proportions in the experimental groups than in the control. It is important to note that MKLS had a more promising fatty acid composition than MVLS, with clearer methane mitigation. Attempts to improve the FA content of milk therefore require an understanding of the interrelationships between dietary lipid supply, ruminal fermentation, and the metabolic changes that occur in the blood, liver, and ultimately in the mammary glands (Brzozowska and Oprządek, 2016). The duration of the experiment can also play an important role. As stated by Wang et al. (2017), the addition of tea saponins to dairy cows' diet led to an increase in UFA content in their milk over the four weeks of treatment, but later the UFA content tended to be similar to the beginning of the treatment, indicating that there may have been adaption to high doses of saponin supplements.

5. Conclusion

We conclude that the ensiled form of the Kometa lucerne variety seems to be a good sourc of saponins, which are capable of effectively reducing methane production. It has been confirmed in both in vitro and in vivo experiments. Unfortunately, the results of these two types of research did not completely coincide, which can be explained by the different conditions prevailing on the microorganisms. Under the conditions of the present in vivo study, we assumed that a high-forage diet containing high levels of Kometa lucerne silage decreased methane production in dairy cows by reducing protozoa and methanogen counts without negatively affecting the basic fermentation parameters. However, no effect was observed on milk production or milk FA composition. This may be because grass silage was only partially substituted by Kometa lucerne silage in the experimental diets. Despite this, Kometa lucerne silage can be successfully used as a dietary component in dairy cows' diets to mitigate methane emission.

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CRediT authorship contribution statement

Martyna Kozłowska: Conceptualization, Methodology, Writing - original draft. **Adam Cieślak:** Investigation, Supervision, Data curation, Writing - review & editing. **Artur Jóźwik:** Investigation, Writing - review & editing. **Mohamed El-Sherbiny:** Methodology, Writing - original draft. **Maciej Gogulski:** Data curation. **Dorota Lechniak:** Data curation. **Min Gao:** Visualization, Validation. **Yulianri Rizki Yanza:** Visualization, Validation. **Mina Vazirigohar:** Data curation. **Malgorzata Szumacher-Strabel:** Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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