The Effect of Zinc and/or Herbal Nutraceuticals on Rumen Fermentation, Microbiota and Histopathology in Lambs

Daniel Petrič
Slovak Academy of Sciences: Slovenska akademia vied
Dominika Mravčáková
Slovak Academy of Sciences
Katarína Kucková
Slovenská akadémia vied: Slovenska akademia vied
Svetlana Kišidayová
Slovak Academy of Sciences: Slovenska akademia vied
Adam Cieslak
Poznan University of Life Sciences: Uniwersytet Przyrodniczy w Poznaniu
Malgorzata Szumacher-Strabel
Poznan University of Life Sciences: Uniwersytet Przyrodniczy w Poznaniu
Haihao Huang
Poznan University of Life Sciences: Uniwersytet Przyrodniczy w Poznaniu
Pawel Kolodziejski
Poznan University of Life Sciences: Uniwersytet Przyrodniczy w Poznaniu
Anna Lukomska
Poznan University of Life Sciences: Uniwersytet Przyrodniczy w Poznaniu
Sylwester Slusarczyk
Wroclaw Medical University: Uniwersytet Medyczny im Piastow Slaskich we Wroclawiu
Klaudia Čobanová
Slovak Academy of Sciences: Slovenska akademia vied
Zora Varadyova (varadyz@saske.sk)
Slovak Academy of Sciences: Slovenska akademia vied https://orcid.org/0000-0003-3675-8641

Research

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Abstract

Background: The aim of this study was to investigate the effect of diets containing organic zinc and a mixture of medicinal herbs on rumen microbial fermentation and histopathology in lambs.

Methods: Twenty-eight lambs were divided into four groups: unsupplemented animals (Control), animals supplemented with organic zinc (Zn, 70 mg Zn/kg diet), animals supplemented with a mix of dry medicinal herbs (Herbs, 100 g DM/d) and animals supplemented with both zinc and herbs (Zn+Herbs). Each lamb was fed a basal diet composed of meadow hay (700 g DM/d) and barley (300 g DM/d). *Fumaria officinalis* L. (FO), *Malva sylvestris* L. (MS), *Artemisia absinthium* L. (AA) and *Matricaria chamomilla* L. (MC) were mixed in equal proportions. The lambs were slaughtered after 70 days of the experiment. The rumen content was used to determine the fermentation parameters *in vitro* and *in vivo* and to quantify the microbes by molecular and microscopic methods. Samples of fresh rumen tissue were used for histopathological evaluation.

Results: Quantitative analyses of the bioactive compounds identified 3.961, 0.654, 6.482 and 12.084 g/kg DM phenolic acids and 12.211, 6.479, 0.349 and 2.442 g/kg DM flavonoids in FO, MS, AA and MC, respectively. The alkaloids content in FO was 6.015 g/kg DM. The effect of diets on values of total gas, methane and *n*-butyrate *in vitro* was observed ($P < 0.046$ and $P < 0.001$, respectively). Relative quantification by real-time PCR revealed a decrease in the total rumen bacterial population in the lambs in the Zn and Zn+Herbs groups ($P < 0.05$). A shift in the relative abundance of *Ruminococcus albus*, *Streptococcus bovis, Butyrivibrio proteoclasticus* and *R. flavefaciens* was observed in the Zn group. Morphological observation showed focal mixed infiltration of inflammatory cells within the lamina propria of the rumen in the Zn+Herbs group.

Conclusion: The effect of the organic zinc and the herbal mix on rumen fermentation parameters *in vitro* was not confirmed *in vivo* because the rumen microbiota of lambs probably adapted to the zinc-supplemented diets. However, long-term supplementation of a diet combining zinc and medicinal herbs can diminish the health of the ruminal epithelium of lambs.

Background

Rumen microbial fermentation of dietary substrates plays the main role in the ability of ruminants to utilize fibrous dietary substrates but is also associated with the methane emissions and excessive nitrogen excretions in manure. Nowadays, modern production systems require the use of different nutraceuticals for optimal production and health in ruminant nutrition. Therefore, it is necessary to understand the ruminal microbiome in all aspects of bacterial (archaeal and eukaryote populations) and in all factors for manipulating it to maximize productivity while decreasing negative environmental impacts [1, 2]. Our previous results showed that replacing 10% of meadow hay with different mixtures of dry medicinal plants could influence fermentation patterns [3, 4]. Recent findings with plant nutraceuticals highlight that the effect of dry medicinal plants depends on the variety and synergy of plant polyphenols and the combination of bioactive compounds, which together have an effect and contribute to a certain pharmacological efficacy [5, 6].

It is well known that zinc plays catalytic, structural and regulatory roles for enzymes, proteins and transcription factors and is thus a key trace element for improving immune functions [7, 8]. Additionally, the bioavailability of zinc from the diet in ruminants depends on the zinc's chemical form, content and interaction with dietary constituents [9, 10]. The most recent requirements and recommendations for dietary zinc in ruminants vary between 40 and 130 mg/kg DM of the complete diet [11]. The organic form of trace elements bound by organic ligands should be more resistant to interactions in the ruminant digestive tract and can be more bioavailable than inorganic sources [12]. The rumen of ruminants allows selective uptake of nutrients generated by intraruminal microbial fermentation because the rumen is covered by a stratified epithelium that consists of leaflike papillae, which greatly increase the absorptive surface area and size [13]. Epithelial surfaces are complex chemical and biological barriers that prevent microbial invasion or potentially harmful pathogens, but they also harbor many beneficial microorganisms [14].

Our recent *in vitro* study showed that a mix of fumitory, mallow, wormwood and chamomile possessed a strong ruminal antioxidant capacity with the potential for inducing desirable changes in the gastrointestinal ecosystem during ruminant fermentation [15]. Ruminal volatile fatty acids (VFAs) *arise* largely from the *fermentation* of dietary carbohydrates and are absorbed through the rumen epithelium; therefore, we hypothesized that this herbal mixture, together with organic zinc, can affect not only rumen fermentation and the microbial population but also rumen histopathology. Our knowledge is also based on our previously published findings showing that a combination of zinc and a special medicinal herbal mix can positively influence the health of lambs with gastrointestinal nematode infection [16]. We aimed to investigate the effects of dietary supplements containing organic zinc and a mixture of medicinal herbs (i.e., *Fumaria officinalis* L., *Malva sylvestris* L., *Artemisia absinthium* L. and *Matricaria chamomilla* L.) on rumen fermentation and the microbial population as well as histopathology in lambs.

Materials And Methods

Lambs, diets and experimental design

The experimental design followed EU standards for the protection of animals under European Community guidelines (EU Directive 2010/63/EU). The Ethical Committee of the Institute of Animal Physiology of the Centre of Biosciences of SAS approved the experimental protocol (resolution

number Ro-4065/18-221/3). Twenty-eight castrated male Improved Valachian lambs at approximately 5 months of age and an initial body weight of 22.6 ± 2.94 kg were housed individually in pens for 30 days for acclimatization to feeding with free access to water. The animals were divided based on their live-weight into four groups (n = 7): unsupplemented animals (Control), animals supplemented with zinc chelate of amino acids hydrate (Zn), animals supplemented with a mix of herbs (Herbs) and animals supplemented with both zinc and a mix of herbs (Zn+Herbs). Each lamb was fed a basal diet composed of meadow hay (700 g DM/d) and barley (300 g DM/d). The aliquots of zinc Availa-Zn 100 EU (Zincpro Corporation, Eden Prairie, MN) were directly mixed with the feeding concentrate for each meal to provide 70 mg Zn/kg diet. Herbs (100 g DM/d) from commercial sources (AGROKARPATY, Plavnica, Slovak Republic) were stable throughout the experiments and contained 33% each of *F. officinalis* (FO), *M. sylvestris* (MS) and *M. chamomilla* (MC) as well as 1% of *A. absinthium* (AA). All experimental groups received diets for 70 days, and all the animals were humanely killed at the end of the experiment on three consecutive days (abattoir of the Centre of Biosciences of SAS, Institute of Animal Physiology, Košice, Slovakia, No. SK U 06018). Ruminal contents, samples for quantification of rumen microbes and samples of the fresh rumen tissues were collected. The animal carcasses were sent to the Department of Pathological Anatomy and Pathological Physiology, University of Veterinary Medicine and Pharmacy in Košice, Slovak Republic.

Experiment *in vitro*

The experiment was carried out using the *in vitro* gas production technique (IVGPT) on batch culture incubations of buffered rumen fluid (RF) incubated for 24 h at 39 °C in anaerobic conditions [17]. The rumen content was collected separately from each lamb of each treatment immediately after slaughter in the abattoir, packed in pre-warmed flasks and transported to the laboratory. The rumen content was squeezed through four layers of cheesecloth and pooled according to diet treatments of donor animals. The pooled RF was purged with CO_2, mixed at a 1:1 ratio with McDougall's buffer [18] and dispensed in volumes of 35 mL into fermentation bottles (120 mL) containing 250 mg (DM basis) of a substrate. Meadow hay (MH) and barley grain (BG) were used as the basic components of the diet (700:300, w/w) with the use of additive Zn (0.025 g/bottle), Herbs (0.025 g/bottle) or both, respectively. Herbs, MH or BG were ground and sieved through a 0.15-0.40 mm screen using a grinder (Molina, MIPAM, České Budějovice, Czech Republic). The *in vitro* experiment was arranged according to a completely randomized design using the four diets (Control, Zn, Herbs, Zn+Herbs) in fermentations with four different rumen fluid inocula (Control, Zn, Herbs, Zn+Herbs) using three replicates (three incubation bottles) for each diet and each inoculum. The experiment was repeated three times within three consecutive days (n = 3 × 3).

Chemical analysis and measurements

The dietary substrates were analyzed in triplicate using standard procedures for dry matter (method no. 967.03), nitrogen (method no. 968.06), crude protein (method no. 990.03) and ash (method no. 942.05) [19]. The acid-detergent fiber (ADF) and neutral detergent fiber (NDF) contents were determined [20] using the FiberCap system (FiberCap ™ 2021/2023, FOSS Analytical AB, Höganäs, Sweden). In the forages, NDF was assayed without a heat-stable amylase and expressed inclusive of residual ash. In the concentrate, NDF was assayed with a heat-stable amylase and expressed inclusive of residual ash. ADF was also expressed inclusive of residual ash. The chemical composition of the dietary substrates is presented in Table 1. The volume of accumulated total gas was determined after 24 h using IVGPT. For methane analysis, 1 mL of gas was collected *via* IVGPT using an air-tight syringe (GASTIGHT Syringes, Hamilton Bonaduz AG, Switzerland) and injected into a gas chromatograph. The VFAs and methane were analyzed by gas chromatography on a PerkinElmer Clarus 500 gas chromatograph (Perkin Elmer, Shelton, USA) [21]. Methane production *in vivo* was calculated based on stoichiometric relationships between VFA composition and methane production [22]. The pH values of the batch cultures were measured using a pH meter (InoLab pH Level 1, Weilheim, Germany). The concentration of ammonia-N was determined in the inocula using the phenol-hypochlorite method [23].

Analysis of flavonoids and phenolic acids

Each of the herb materials (100 mg) – *F. officinalis*, *M. sylvestris*, *A. absinthium,* and *M. chamomilla* were ground to a fine powder and extracted three times with 80% methanol (MeOH) for 30 min at 40 °C. The extracts were evaporated to dryness and were dissolved in 2 mL of Milli-Q water (acidified with 0.2% formic acid) and purified by Solid Phase Extraction (SPE) using Oasis HLB 3cc Vac Cartridge, 60 mg (Waters Corp., Milford, MA). The cartridges were washed with 0.5% MeOH to remove carbohydrates and then washed with 80% MeOH to elute phenolics. The phenolic fraction was re-evaporated and dissolved in 1 mL 80% MeOH (acidified with 0.1% formic acid). The sample was then centrifuged (23,000 × g, 5 min) before spectrometric analysis. All analyses were performed in triplicate for three independent samples and stored in a freezer at – 20 °C before analysis.

Analysis of alkaloids

Herbal materials of FO were ground to a fine powder, and 100 mg were extracted with 0.5M H_2SO_4 for 20 min in an ultra-bath at 25 °C; the procedure was then repeated in the same manner and the filtrates combined. Then the filtrates were adjusted to pH 9-10 by 1M NaOH and separated with $CHCl_3$. The lower organic layer was collected and evaporated to dryness under reduced pressure, then dissolved in 80% MeOH for further analysis.

Ultra-High-Resolution Mass Spectrometry (UHRMS)

The bioactive compounds of the individual medicinal herbs (FO, MS, AA and MC) were determined using UHRMS on a Dionex UltiMate 3000RS (Thermo Scientific, Darmstadt, Germany) system with a charged aerosol detector interfaced with a high-resolution quadrupole time-of-flight mass spectrometer (HR/Q-TOF/MS, Compact, Bruker Daltonik GmbH, Bremen, Germany). The mix of herbs metabolome was chromatographically separated on a Kinetex C18 column (2.1 × 100 mm, 2.6 µm, Phenomenex, USA), with mobile phase A consisting of 0.1% (v/v) formic acid (FA) in water and mobile phase B consisting of 0.1% (v/v) FA in acetonitrile. A linear gradient from 7% to 30% of phase B in phase A over 20 min was used to separate phenolic compounds with a short 0.3 min calibration segment from 0 to 0.5 min. The flow rate was 0.3 mL/min and the column was held at 25 °C. Spectra were acquired in negative-ion mode over a mass range from m/z 100 to 1500 with 5 Hz frequency. Operating parameters of the ESI ion source were as follows: capillary voltage 3 kV, dry gas flow 6 L/min, dry gas temperature 200 °C, nebulizer pressure 0.7 bar, collision radio frequency 700.0 V, transfer time 100.0 µs, and pre-pulse storage 7.0 µs. Ultrapure nitrogen was used as the drying and nebulizer gas, and argon was used as the collision gas. The collision energy was set automatically from 15 to 75 eV depending on the m/z of fragmented ion. Acquired data were calibrated internally with sodium formate introduced to the ion source at the beginning of each separation via a 20 µL loop. The processing of spectra was performed with Bruker DataAnalysis 4.3 software (Bruker Daltonik GmbH, Bremen, Germany). The amount of the particular phenolic acids in the samples were calculated as the chlorogenic acid (CAS 327-97-9, 3-Caffeoylquinic acid) equivalent, and hyperoside (CAS 482-36-0, quercetin 3-galactoside) was used for calculating the amounts of identified flavonoids. Stock solutions of hyperoside and chlorogenic acid were prepared in MeOH at concentrations of 3.1 and 4.1 mg/mL, respectively, and kept frozen until used. Calibration curves for these two compounds were constructed based on seven concentration points (from 500 to 3.9 µg/mL).

The total content of alkaloids was determined as the chelidonine (CAS 476-32-4) equivalent from the calibration curves based on seven concentration points of chelidonine (from 200 to 1.2 µg/mL). Separation of the alkaloids was done with the same HPLC condition as with the phenolic compounds, with one exception: positive-ion mode was used in the acquired spectra in auto MSMS. All analyses were performed in triplicate.

Quantification of rumen microbes

Samples for counting ciliate protozoa were fixed with an equal volume of an 8% solution of formaldehyde and were counted and identified microscopically according to [24]. For bacterial quantification, DNA from the ruminal samples was isolated using a Mini Bead-Beater (BioSpec, Bartlesville, OK, USA) for cell lysis [25], followed by purification (QIAamp DNA Stool Mini Kit; Qiagen, Hilden, Germany). DNA concentrations and quality were measured with a NanoPhotometer® NP80 (Implen GmbH, München, Germany). For relative quantification real-time PCR of eubacteria and archaea, we used the PCR primers according to the study [26]. The relative 16S rRNA gene copy abundance was expressed as an arbitrary unit (AU) relative to the total bacterial gene copy abundance of the Control.

Histological parameters

Samples of fresh tissues of rumen were washed in phosphate buffer solution (0.1 M, pH 7.4), put in a plastic container and fixed in a 10% buffered FA solution as spread tissues pieces on flat polystyrene. The fixed material was processed with a series of reagents and embedded in paraffin blocks (Paraplast PLUS, Leica, Buffalo Grove, USA), which were then cut with a rotary microtome into 3.5 µm thick sections. Slides with a paraffin section were automatically stained with hematoxylin and eosin (HE, Varistain Gemini Thermo Scientific, UK). Histological evaluation was performed using an Axio Lab. 1 microscope (Carl Zeiss) equipped with an Axiocam ERc5s digital camera (Carl Zeiss, Jena, Germany). Pictures were analyzed and recorded by computer software (ZEN 2.3 (blue edition), Carl Zeiss Microscopy GmbH, 2011).

Hematological analyses

Samples of blood were collected from each lamb at D0, D35 and D70. They were taken from the jugular vein using a 21-gauge needle and syringe and collected into microtubes containing 1.6 mg/mL EDTA-K3 (Sarstedt AG & Co, Nümbrecht, Germany). Hematological parameters were determined immediately by an automated hematological analyzer (Abbott CELL-DYN 3700, Global Medical Instrumentation, Inc., Ramsey, USA).

Data analysis

Statistical analysis of the data was performed using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA, Version 8.3.0 (538), 2019). Data on ruminal fermentation parameters and ciliate populations *in vitro* were analyzed by two-way ANOVA. The model included effects for diets, inocula and Diet × Inoculum interaction. Statistical analysis of the hematological parameters used analysis of variance as a repeated measure mixed model that represented the four animal groups and sampling days. Data on rumen fermentation parameters and microbial population *in vivo* were evaluated by multiple comparisons of one-way ANOVA using Dunnett's multiple comparisons test. The total and differential counts of the rumen ciliates were analyzed using the nonparametric Kruskal-Wallis test. The effects were determined to be significant at $P < 0.05$.

Results

Phytochemical substances in medicinal herbs

The phytochemical substances of FO contained 12.211 g/kg DM of flavonoids, 3.961 g/kg DM of phenolic acids (Table 2) and 6.015 g/kg DM of alkaloids (Table 3). The phytochemical compounds of MS contained 6.479 g/kg DM of flavonoids and 0.654 g/kg DM of phenolic acids (Table 2). The AA contained 0.349 g/kg DM of flavonoids and 6.482 g/kg DM of phenolic acids (Table 2). Quantitative analyses of the bioactive compounds in MC identified 2.442 g/kg DM of flavonoids and 12.084 g/kg DM of phenolic acids (Table 2).

Effect of dietary substrates on ruminal fermentation *in vitro*

The effect of dietary substrate and inoculum on ruminal fermentation parameters *in vitro* is shown in Table 4. The inoculum of the donor animals affected the values of all fermentation parameters ($P < 0.001$). There was a significant effect of diet on total gas ($P < 0.046$) and methane ($P < 0.001$) production; their values were lower in the Zn diet. The values of *n*-butyrate also varied among the dietary treatments ($P < 0.001$), with the higher concentration of *n*-butyrate occurring for the Zn and Zn+Herbs diets compared to the Control. A significant effect of different ruminal inocula on the protozoal population of lambs *in vitro* was also observed (Table 5).

Effect of zinc and herbal diets on rumen fermentation and microbiota in lambs

No effects ($P > 0.05$) of dietary supplements on rumen fermentation parameters were observed in the lambs (Table 6). There were also no significant differences ($P > 0.05$) between the experimental groups in the number of rumen ciliated protozoa expressed as count per g of wet ruminal content (wRC) or as count per gram of DM of ruminal content, respectively, in the lambs (Table 7). The lambs contained three different ciliate populations. The mixed A-B type population was determined in 19 animals (68%), with the presence of *Polyplastron multivesiculatum* (100%), *Epidinium ecaudatum caudatum* (84%) and *Ophryoscolex caudatus tricoronatus* (53%). Seven animals (25%) harbored the A type population, with the presence of only *P. multivesiculatum* (100% prevalence) and *O. caudatus tricoronatus* (29% prevalence), and two animals (7%) harbored the B type population, with *E. ecaudatum caudatum* (100% prevalence) occurring but not *Polyplastron* or *Ophryoscolex*. The species *Dasytricha ruminantium*, *Isotricha* spp. (*I. intestinalis, I. prostoma*) and *Entodinium* spp. were present in all animals. The total bacterial population (Fig. 1A) of lambs fed with Zn and Zn+Herbs diets decreased ($P < 0.05$); however, *Ruminococcus albus* (Fig. 1C), *Streptococcus bovis* (Fig. 1D) and *Butyrivibrio proteoclasticus* (Fig. 1E) had higher relative abundance in the group fed the Zn diets ($P < 0.05$ or $P < 0.01$, respectively). In contrast, the relative abundance of *Ruminococcus flavefaciens* (Fig. 1K) in the Zn group was lower than in other groups ($P < 0.05$). Also, *Fibrobacter succinogenes* (Fig. 1F) abundance was lower in the Herbs group compared with the other groups ($P < 0.05$). The other microbial populations, such as *Archaea* (Fig. 1B), *Butyrivibrio fibrisolvens* (Fig. 1G), the genus *Prevotella* (Fig. 1H), *Clostridium aminophilum* (Fig. 1I) and *Megasphaera elsdenii* (Fig. 1J), did not differ among the treatment groups ($P > 0.05$).

Effect of diets on morphological parameters of ruminal papillae

The rumen papillae Control and Zn groups had a normal histological appearance (Fig. 2 A, B). A diverse histological structure in the size of the papillae in the Herbs group (Fig. 2 C) and in both the type and number of keratinized epithelial cells in the Zn+Herbs groups (Fig. 2 D) were observed. Focal mixed infiltration of inflammatory cells in single papilla within the epithelial layer was observed in two animals fed the Herbs diet and within the lamina propria in most animals fed the Zn+Herbs diet.

Hematological parameters

The red blood cell count, hemoglobin level and hematocrit were not influenced by time, treatment or the treatment × time interaction ($P > 0.05$) (Table 8). Time affected the mean corpuscular volume levels ($P < 0.001$). Treatment and time affected neutrophil levels ($P < 0.05$), and time also affected the counts of lymphocytes and eosinophils ($P < 0.001$ or $P < 0.05$, respectively).

Discussion

Our previous results showed that the addition of zinc to the diet of the lambs does not negatively influence microbial activity in their rumen and large intestine [4]. Additionally, a mix of herbs containing FO, MS, AA and MC possesses a strong ruminal antioxidant capacity and reduces gastrointestinal methane and ammonia concentration *in vitro* [15]. However, to the best of our knowledge, the present experiment is the first study where the interactions between organic zinc and herbal supplementation were studied by combining phytochemical, physiological, microbiological and histopathological measurements in the rumen of lambs.

In the present experiment, the phytochemical substances of FO contained flavonoids, alkaloids and phenolic acids mainly of quercetin-O-Hex-Hex (2.384 g/kg DM), quercetin O-Pen-Hex (3.5 g/kg DM), fumariline (1.728 g/kg DM), fumaricine (0.102 g/kg DM) and caffeoylmalic acid (1.212 g/kg DM). The flavonoid (flavonol) quercetin possesses various antioxidative and anti-inflammatory effects as well as metabolic health-promoting properties [27]. Also, both isoquinoline alkaloids fumariline and fumaricine contribute to the important pharmacological activities of FO [28]. Additionally, the phenolic compound caffeoylmalic acid is able to protect protein from degradation in ruminants that utilize forage protein [29]. The flavonoids delphinidin 5-glucoside 3-lathyroside (1.644 g/kg DM) and apigenin-O-Hex (1.56 g/kg DM) were identified in the

greater concentration in MS. The health-promoting effect of apigenin O-Hex with therapeutic potential has already been highlighted [30]; however, flavonoids generally possess beneficial biochemical properties with predominantly protective roles against many diseases [31]. The phytochemical substances of AA contained mainly phenolic acids, including chlorogenic acid (3.416 g/kg DM) and 1,5-dicaffeoylquinic acid (2.124 g/kg DM), which possess antibacterial, anthelmintic, anti-inflammatory and antioxidant biological activities *in vitro* and *in vivo* [32, 33]. Similarly, MC contained phenolic acids (12.084 g/kg DM), mainly methyl 4-O-beta-d-glucopyranosylcaffeate (3.202 g/kg DM), with well-known antioxidant activity [34], and more caffeoylquinic acid derivatives which have anti-inflammatory biological activities [35].

The mix of the dry medicinal herbs used in the present experiment has great concentrations of flavonoids especially of quercetin (7.6 mg/g DM) [15]. In cows, after intra-ruminal application (10 and 50 mg/kg BW), quercetin is an extensively degraded flavonoid by ruminal microbiota without negative effects on ruminal fermentation [36]. However, the administration of some flavonoids with antimicrobial properties can affect the gastrointestinal microbiota. Under *in vitro* conditions in the present study, the effect of diets (i.e., Zn, Herbs, and Zn+Herbs) was shown only in the values of total gas, methane and *n*-butyrate. Some research studying the effects of flavonoids as potential diet additives for ruminants (e.g., quercetin, myricetin, kaempferol, rutin, etc.) are inconsistent due mainly to their potential antimicrobial effects [37, 38, 39]. However, the effect of ruminal inocula *in vitro* (Table 5) was significant for all fermentation parameters and protozoa species. The inoculum effect can be ascribed to the diverse rumen ciliate populations and companion bacterial populations of the experimental animals. The results in Table 5 show the number of protozoa that were found in each experimental group. However, *Ophryoscolex* and *Epidinium* were not present in every treatment group. Also, a prevalence of the mixed A-B type ciliate population was observed (19 animals). This probably points to the gradual change of the B type population into the dominant A type population in lamb floc during the experiment, as Polyplastron predate on Epidinium until it disappears from the ciliate population [24]. A significant effect of the inocula in the present experiment was identified mainly in *Dasytricha* species. This was probably caused by long-term dietary zinc or herbal supplementation to the diet of experimental lambs, which can influence the composition of the eubacterial community and the enzymatic activities of rumen microorganisms especially amylolytic and cellulolytic [40, 41].

No dietary effects (i.e., Zn, Herbs, and Zn+Herbs) were found in the present study on either fermentation parameters or protozoal populations in lambs. This probably points to the relatively low content of the anti-methanogenic phytochemical substances or the adaptation of microbiota to both herbal [42, 43] and/or zinc diets [44]. Herbal diets can influence the rumen microbiome, the kinetics of fermentation and the response and adaptation to anti-methanogenic compounds and diets that lead sometimes to the inconsistent efficacy of phytochemical substances [45]. Zn is also involved in a wide assortment of physiological processes; therefore, nutrient digestibility may be affected by supplemental Zn, which is incorporated into enzymes throughout the body and is critical for most metabolic processes in ruminants [46]. It is known that zinc in the nutrition of ruminants can significantly influence ruminal fermentation [47, 48]. While low doses of zinc (20-70 mg Zn/kg diet) have a weak effect on rumen fermentation [4, 10], higher doses (250-1142 mg Zn/kg diet) can affect the rumen protozoal population and protein degradation [49]. Despite the fact that zinc retention in lambs can be similar regardless of the source of Zn or its concentration [46], the beneficial effect of zinc (i.e., antioxidant, anti-inflammatory or antiapoptotic, respectively) strongly depends on both of these factors, and too much or too little zinc in diets can lead to the opposite effect [50, 51].

In the present experiment, the amount of starch in all the diets fed to the lambs was similar and therefore probably influenced the growth efficiency of the majority of rumen ciliates in the same way [52]. However, the total bacteria of the Zn and Zn+Herbs groups were lower than the Control and Herbs groups. The relative abundance of the cellulolytic bacteria *R. albus*, amylolytic *S. bovis* and polysaccharide-degrading *B. proteoclasticus* increased and the abundance of cellulolytic *R. flavefaciens decreased in the Zn group* compared to the other diet groups. *Certain bacterial species were probably enriched by Zn supplementation in the diet at the expense of total bacterial abundance* [44]. These changes in total bacteria and relative abundance of some bacteria in the Zn groups were not accompanied by changes in VFAs in rumen fermentation *in vivo*. This points to a direct effect on rumen microbiota due to an interaction with crude protein rather than the benefits Zn supplementation, which could be exerted solely on the host organism [8]. However, the decrease in relative abundance of cellulolytic bacteria (i.e., *F. succinogenes* and *R. flavefaciens*) in the Zn or Zn+Herbs groups, respectively, probably also lowered the substrate digestibility *in vitro* in these groups (Table 4). It seems that the rumen microbiota has its requirements for zinc supplementation which does not cause a major shift in the rumen bacterial community and is without negative consequences for digestion and animal health [44]. Additionally, bacterial phylotypes are known to contribute to differences in feed efficiency and host productivity and can or needn't depend on diets [53]. Concerning the Herbs group, the relative abundance of the starch-fermenting bacteria *S. bovis* and in particular the cellulolytic bacteria *F. succinogenes* was low compared to the Control and Zn groups, probably due to the antimicrobial activity of some flavonoids, which can increase competition among bacteria [39, 54].

Ruminal VFAs are absorbed through the ruminal epithelium, and the rate of absorption depends on the VFA concentration, rumen papillae surface area and the availability of transport proteins [55, 56]. Rumen papillae of lambs from the Control and Zn groups presented a normal histological appearance with a diverse histological structure concerning the size of the papillae, their type and the number of keratinized epithelial cells. The external layer of vesiculated keratinized cells of the ruminal epithelium is the absorption barrier for the transport of molecules from the rumen into the blood [57]. In the Zn group, the stratum corneum contained several layers of vacuolated horn cells with a large amount of keratin in the cytoplasm and cellular organelles. The lambs in the Herbs and Zn+Herbs groups had rumen papillae with diverse histological structures related mostly to the size of the papillae, type and number of keratinized epithelial cells. Butyrate is known to stimulate ruminal papillae development [58]. However, a higher molar proportion of *n*-butyrate was found only in the Zn and Zn+Herbs groups *in vitro*. The amount of ruminal VFA

absorption can decrease with increasing ruminal parakeratosis [59], and a physical barrier could reduce the transport of VFAs to the deepest layers of the epithelium. The health of the ruminal epithelium was probably diminished because the focal mixed infiltration of inflammatory cells in single papillae within the epithelial layer and the lamina propria was observed in lambs from the Herbs or Zn+Herbs groups, respectively. However, the hematological parameters of the lambs were not affected by treatment. It seems that the 70 days application of the Herbs was too long.

Nutraceuticals provide health benefits beyond basic nutrition. Although the vast number of naturally occurring health-enhancing substances are of herbal origin, there are also many physiologically active components, such as trace elements, that play an important role in animal health promotion. Limited research is available on the effects of nutraceuticals like zinc and/or herbs on blood profiles [16, 60]. Based on pharmacological and clinical research *M. sylvestris, A. absinthium* and *M. chamomilla* are promising herbs for the treatment of gastrointestinal disorders [61]. Additionally, the supplementation of the diet with *A. absinthium* can also enhance the growth rate of lambs with higher weight gains [62]. However, it seems that different nutrients generally can improve the absorptive capability of the ruminal epithelium, protect the epithelium against the damage as well as alter the expression of genes regulating rumen epithelial morphology [63, 64, 65].

Conclusions

It can be concluded that the ability of organic zinc (70 mg Zn/kg diet) and herbal supplementation (100 g DM/d) to influence rumen fermentation and the composition of rumen microbiota *in vitro* has not been confirmed *in vivo*. No effects of dietary supplements on rumen fermentation parameters and the protozoal population of lambs were found, probably because during the 70 days of feeding the lambs adapted to diets with a decrease in total bacteria and a shift in the relative abundance of cellulolytic and amylolytic bacteria in the zinc experimental group. However, our results also point out that long-term dietary supplementation with organic zinc combined with a mix of medicinal herbs can probably diminish the health of the ruminal epithelium; therefore, more *in vivo* experiments are *necessary.*

List Of Abbreviations

A: Acetate; AA: *Artemisia absinthium* L.; ADF: Acidic-detergent fiber; ANOVA: Analysis of variance; BG: Barley grain; CP: Crude-protein; DM: Dry matter; FA: Formaldehyde; FO: *Fumaria officinalis* L.; HPLC: High-performance liquid chromatography; iB: Iso-butyrate; iV: Iso-valerate; IVDMD: *In vitro* dry DM digestibility; IVGPT: *In vitro* gas production technique; MC: *Matricaria chamomilla* L.; MeOH: Methanol; MH: Meadow hay; MS: *Malva sylvestris* L.; MS^2: Tandem mass spectrometry; N: Nitrogen; nC: N-caproate; NDF: Neutral-detergent fiber; NS: Not significant; nB: N-butyrate; nV: N-valerate; P: Propionate; RF: Rumen fluid; SD: Standard deviation; SEM: Standard error of means; UHRMS: Ultra-high resolution mass spectrometry; VFAs: Volatile fatty acids

Declarations

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Author's contributions

DP, DM, and KK collected data and provided laboratory analysis. DP and DM performed the *in vitro* experiments. PK, HH, and DM performed rumen microbial quantification and statistical analyses. SK conducted ciliates population analysis, statistical analyses, and reviewed the manuscript. AL conducted histological analyses and evaluations. SS provided an analysis of the bioactive compounds. AC and MSS provided data curation and reviewed the manuscript. KC and ZV designed the study protocol and supervised the research. ZV interpreted the data and wrote the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials

Data are available upon reasonable request to the corresponding author.

Ethics approval

The experimental design followed EU standards for the protection of animals under European Community guidelines (EU Directive 2010/63/EU). The Ethics Committee at the Institute of Animal Physiology of the Centre of Biosciences of the SAS approved the experimental protocol (resolution number Ro-3355/16-221).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Institute of Animal Physiology, Centre of Biosciences of the Slovak Academy of Sciences, Košice, Slovak Republic. [2]Department of Animal Nutrition, Poznan University of Life Sciences, Poznan, Poland. [3]Department of Animal Physiology, Biochemistry and Biostructure, Poznan University of Life Sciences, Poznan, Poland. [4] Department of Preclinical Sciences and Infectious Diseases, Poznan University of Life Sciences, Poznan, Poland. [5]Department of Pharmaceutical Biology and Botany, Wroclaw Medical University, Wroclaw, Poland.

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Tables

Table 1 Chemical composition of the dietary ingredients

	Meadow hay	Barley	Mixture of herbs	Availa-Zn100	Barley + Availa-Zn100
Dry matter, g/kg	894	875	874	976	869
Main chemical composition, g/kg DM					
Neutral-detergent fibre	535	277	390	286	516
Acid-detergent fibre	345	108	217	237	138
Nitrogen	23	22	33	34	26
Crude protein	144	137	206	212	165
Ash	77	25	127	361	27

Table 2 Content of greater bioactive compounds in *F. officinalis, M. sylvestris, A. absinthium* and *M. chamomilla*

RT, min	UV, nm	*m/z*[M-H]$^-$	MS^2	MS^2 fragments	Formula	Compounds	Flavonoids, g/kg DM	Phenolic acids, g/kg DM
Fumaria officinalis								
7.00	250/326	295/046	179/0338		$C_{13}H_{12}O_8$	Caffeoylmalic acid		1.212
7.80	227/315	163/0395	119/0499		$C_9H_8O_3$	O-Coumaric acid		0.742
9.00	255/352	625/1398	301/0337		$C_{27}H_{30}O_{17}$	Quercetin-O-Hex-Hex	2.384	
9.40	255/352	595/1287	301/0339		$C_{26}H_{28}O_{16}$	Quercetin O-Pen-Hex	3.500	
9.90	252/351	609/1472	300/0279	285/0391	$C_{27}H_{30}O_{16}$	Isoquercitrin O-Dhex		0.934
10.20	255/354	463/0882	301/0337		$C_{21}H_{20}O_{12}$	Quercetin O-Hex	1.706	
10.90	221/329	593/1520	285/0397		$C_{27}H_{30}O_{15}$	Kaempferol-3-O-rutinoside	0.464	
11.50	255/365	639/1561	315/0504		$C_{28}H_{32}O_{17}$	Isorhamnetin-O-Hex-Hex	0.558	
						Total contents:	12.211	3.961
Malva sylvestris								
7.00	523	757/1846	347/0761	329/261/509	$C_{32}H_{39}O_{21}$	Delphinidin 5-glucoside 3-lathyroside	1.644	
7.90	308	163/0381	119/0502		$C_9H_8O_3$	Coumarinic acid		0.468
10.00		609/1458	301/0330		$C_{27}H_{31}O_{16}$	Quercetin-3-O-rutinoside	0.395	
10.20	268/343	447/0928	285/0386		$C_{21}H_{20}O_{11}$	Kaempferol-O-Hex	0.494	
11.40	268/336	431/0978	269/0435		$C_{21}H_{20}O_{10}$	Apigenin-O-Hex	1.560	
						Total contents:	6.479	0.654
Artemisia absinthium								
4.10	215/325	353/0877	191/0567	179/161/135	$C_{16}H_{18}O_9$	Chlorogenic acid		3.416
11.00		515/1193	353/0867	191/179/135	$C_{25}H_{24}O_{12}$	1.5-Dicaffeoylquinic acid		2.124
11.20		653/1719	345/0595	330/302	$C_{29}H_{34}O_{17}$	Spinacetin 3-rutinoside	0.241	
11.70		515/1192	353/0869	173/179/191/155	$C_{25}H_{24}O_{12}$	4.5-Dicaffeoylquinic acid		0.610
						Total contents:	0.349	6.482
Matricaria chamomilla								
4.30	215/3	353/0877	191/0567		$C_{16}H_{18}O_9$	3-O-Caffeoylquinic acid		1.777
9.00	235/290/319	355/1029	193/049	149	$C_{16}H_{20}O_9$	Methyl 4-O-beta-d-glucopyranosyl caffeate		3.202
9.70	255/4	463/0879	301/0337	151	$C_{21}H_{20}O_{12}$	Quercetin O-Hex	0.199	
10.30	257/4	447/0920	285/0386		$C_{21}H_{20}O_{11}$	Kaempferol O-Hex	1.363	
10.70	217/291/325	515/1189	353/0877	179/191	$C_{25}H_{24}O_{12}$	3.5-		0.824

						Dicaffeoylquinic acid		
11.00	217/291/325	515/1197	353/0869	191/179	$C_{25}H_{24}O_{12}$	1.5-Dicaffeoylquinic acid		3.016
11.40	266/3	431/0976	269/0434		$C_{21}H_{20}O_{10}$	Apigenin O-Hex	0.150	
11.70	215/290/325	515/119	353/0868	173/179/191	$C_{25}H_{24}O_{12}$	4.5-Dicaffeoylquinic acid		0.851
14.40	218/268/339	473/1085	269/0427	406	$C_{23}H_{22}O_{11}$	Apigenin -O-(Hex-Ac)	0.210	
						Total contents:	2.442	12.084

Table 3 Content of the alkaloids with a greater concentration identified in *Fumaria officinalis*

RT, min	UV, nm	*m/z*[M-H]$^-$	MS^2	MS^2 fragments	Formula	Compounds	Alkaloids, g/kg DM
7.70	272	354/1366	305/0811	279/233/323/336	$C_{20}H_{19}NO_5$	Parfumine	0.884
8.40	280	328/1572	265/0865	237/297/313/178	$C_{19}H_{21}NO_4$	Cularimine	0.102
8.50	288	370/1678	291/1029	263/352/337	$C_{21}H_{23}NO_5$	Fumaricine	0.102
8.90	285	326/1410	311/1172	277/294/251/178	$C_{19}H_{19}NO_4$	Cheilanthifoline	0.231
9.20	286	354/1360	275/0713	336/247	$C_{20}H_{19}NO_5$	Chelidonine	0.154
9.40	289	398/1621	338/1397	277/323/249	$C_{22}H_{23}NO_6$	Not determined	0.530
10.20	289	354/1362	275/0692	247/293/206	$C_{20}H_{19}NO_5$	Protopine	0.873
10.60	288	354/1727	206/1139	275/311/338/292	$C_{21}H_{23}NO_4$	Protopine type	0.367
10.80	271	352/1193	279/0647	309/321/263/251	$C_{20}H_{17}NO_5$	Fumariline	1.728
11.20	288	324/1230	249/0764	307/277/219/176	$C_{19}H_{17}NO_4$	Stylopine	0.785
						Total contents:	6.015

Table 4 Effect of different rumen inocula and different diets on the fermentation parameters *in vitro* (*n* = 9)

Inocula	Diet	pH	NH_3-N, mg/L	Gas, mL/g	CH_4, mM	IV DMD[b]	VFA[c]	A[d]	P[d]	iB[d]	nB[d]	iV[d]	nV[d]	nC[d]
Control	Control	6.84	217	229	2.74	574	46.7	66.8	14.9	0.97	13.9	1.81	1.38	0.28
	Zn	6.92	192	222	2.80	518	43.9	66.5	15.2	0.84	14.3	1.60	1.37	0.25
	Herbs	6.91	214	240	2.88	581	46.6	67.0	15.0	0.85	13.8	1.71	1.36	0.31
	Zn+Herbs	6.87	198	227	2.97	502	46.3	66.8	15.3	0.73	14.0	1.54	1.42	0.33
Zn	Control	6.96	305	233	3.06	341	43.8	65.2	15.3	1.55	13.0	2.79	1.75	0.40
	Zn	6.96	301	218	2.62	325	41.1	64.5	15.6	1.55	13.6	2.70	1.74	0.37
	Herbs	6.91	330	250	3.61	347	45.6	65.3	15.5	1.57	12.8	2.73	1.77	0.34
	Zn+Herbs	6.82	273	231	3.31	306	44.0	65.3	15.8	1.27	13.2	2.41	1.69	0.34
Herbs[a]	Control	6.99	248	218	2.66	525	36.2	66.9	16.1	1.10	11.9	2.16	1.59	0.25
	Zn	6.98	214	198	1.73	492	37.8	65.8	15.9	1.08	13.1	2.11	1.65	0.28
	Herbs	6.95	222	216	2.66	554	41.5	66.8	16.4	1.07	11.9	2.05	1.55	0.26
	Zn+Herbs	6.98	216	211	2.47	515	39.5	66.3	16.2	1.05	12.7	1.95	1.58	0.29
Zn+Herbs	Control	7.06	182	184	2.68	585	35.0	65.7	16.7	1.11	12.8	1.95	1.54	0.22
	Zn	7.05	184	173	2.28	575	32.5	64.7	16.3	1.07	14.1	1.94	1.66	0.23
	Herbs	7.04	204	178	2.76	622	35.9	65.8	16.6	1.08	12.9	1.93	1.57	0.21
	Zn+Herbs	7.06	197	180	2.52	571	34.1	65.0	16.7	1.01	13.6	1.83	1.69	0.23
SEM		0.037	24.4	8.65	0.186	39.7	2.60	0.564	0.364	0.104	0.289	0.199	0.065	0.028
Significance of the effects:														
Diet		0.512	0.606	0.046	0.001	0.316	0.256	0.159	0.857	0.211	0.001	0.478	0.771	0.730
Inoculum		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Diet × Inoculum		0.457	0.985	0.942	0.299	0.996	0.999	0.999	0.997	0.983	0.877	0.999	0.877	0.600

Table 5 Effect of different rumen inocula and different diets on the protozoal population *in vitro* (*n* = 9)

Inocula	Diet	*Dasytricha ruminantium*, n/mL	*Isotricha* spp., n/mL	*Polyplastron multivesiculatum*, n/mL	*Entodinium,* 10^3 /mL	Total protozoa, 10^3 /mL
Control	Control	850	1010	330	368	378
	Zn	1320	1140	350	336	347
	Herbs	1450	1230	480	329	338
	Zn+Herbs	2510	1280	570	334	343
Zn	Control	2290	1880	580	374	380
	Zn	2330	2020	710	401	407
	Herbs	2430	1830	710	425	430
	Zn+Herbs	2750	1900	690	395	401
Herbs[a]	Control	3270	800	310	321	326
	Zn	3730	640	250	292	297
	Herbs	3204	530	210	314	318
	Zn+Herbs	3890	520	260	292	297
Zn+Herbs	Control	2440	1120	180	313	318
	Zn	2610	1620	140	350	357
	Herbs	2060	1040	130	327	332
	Zn+Herbs	2720	1670	250	329	335
SEM		111.5	81.8	29.0	9.1	9.2
Significance of the effects:						
Diet		0.073	0.734	0.563	0.982	0.982
Inoculum		< 0.001	< 0.001	< 0.001	0.004	0.004
Diet × Inoculum		0.799	0.933	0.870	0.979	0.982

[a] Mixture of dry fumitory, mallow, wormwood and chamomile

Table 6 Effect of zinc and herbs on rumen fermentation parameters in lambs (*n* = 7)

Item	Control	Zn	Herbs	Zn+Herbs	SD	*P*-value
pH	6.84	6.95	6.89	7.05	0.245	0.468
Ammonia, mg/L	110	129	121	102	38.5	0.629
Methane, mM	0.376	0.382	0.378	0.381	0.071	0.832
Total VFA, mM	53.9	42.6	47.2	37.3	13.9	0.191
Acetate, mol%	69.1	69.1	69.8	68.3	2.04	0.663
Propionate, mol%	13.8	13.9	14.5	14.5	1.55	0.753
n-Butyrate, mol%	13.5	12.1	11.8	12.5	1.59	0.240
iso-Butyrate, mol%	1.03	1.73	1.10	1.65	0.879	0.356
n-Valerate, mol%	0.914	0.910	0.936	1.03	0.221	0.717
iso-Valerate, mol%	1.33	2.00	1.51	1.77	0.822	0.478
n-Caproate, mol%	0.300	0.267	0.263	0.204	0.119	0.546
A:P	5.10	5.04	4.83	4.77	0.567	0.673

Table 7 Effect of zinc and herbs on the number of rumen ciliated protozoa in lambs (*n* = 7)

Genus/treatment	Control	Zn	Herbs	Zn+Herbs	SD	*P*-value
Dasytricha sp., c/g wRC[a]	4 754	5 128	6 717	8 619	5004	0.4333
Isotricha spp., c/g wRC	2 280	3 132	2 656	2 043	1581	0.4170
Polyplastron spp., c/g wRC	2 571	2 723	2 311	663	1445	0.0638
Epidinium spp., c/g wRC	15 905	160	3 055	13 612	9558	0.6615
Entodinium spp., c/g wRC	465 316	485 455	412 898	457 362	93530	0.3916
Total protozoa, c/g wRC	489 413	500 892	427 987	483 593	95191	0.4540
Dasytricha sp., c/g DM[b]	492	609	701	904	503	0.5291
Isotricha spp., c/g DM	301	401	327	241	228	0.2754
Polyplastron spp., c/g DM	234	333	291	75	216	0.1326
Epidinium spp., c/g DM	2258	18	274	1566	1123	0.0441
Entodinium spp., c/g DM	63146	59933	49355	52791	17298	0.4904
Total protozoa, c/g DM	66430	61914	50987	55729	17936	0.4002

[a] Expressed as count per g of wet ruminal content (wRC); [b] Expressed as count per gram of dry matter (DM) of ruminal content

Table 8 Effect of zinc and herbs on the hematological parameters of lambs (*n* = 7)

Parameter	Day	C	Zn	Herbs	Zn+Herbs	SD	Significance of effects:		
							Treatment	Time	Treatment × time
Red blood cells, T/L	0	11.1	10.8	10.8	11.0	0.324			
	35	10.5	10.5	10.7	10.4	0.863	NS	NS	NS
	70	10.1	10.8	10.4	9.47	1.28			
Hemoglobin, g/L	0	102.8	99.1	96.2	98.0	7.08			
	35	97.8	99.2	101.3	98.6	7.56	NS	NS	NS
	70	82.7	100.0	100.6	98.9	15.8			
Hematocrit, L/L	0	0.194	0.218	0.214	0.216	0.028			
	35	0.228	0.230	0.229	0.222	0.014	NS	NS	NS
	70	0.226	0.236	0.229	0.208	0.025			
Mean corpuscular volume, fL	0	20.0	20.3	19.9	19.8	1.15			
	35	21.8	21.8	21.6	21.5	1.28	NS	***	NS
	70	22.6	22.0	22.0	22.1	1.14			
Total leukocytes, g/L	0	8.26	8.70	7.67	6.60	2.34			
	35	8.33	8.45	8.25	7.70	1.69	NS	NS	NS
	70	8.55	6.54	8.83	8.59	1.33			
Neutrophils, g/L	0	2.60	3.90	2.56	2.09	1.36			
	35	2.79	3.00	3.03	2.37	0.897	NS	NS	*
	70	3.11	2.01	4.04	3.26	0.953			
Lymphocytes, g/L	0	3.14	2.50	2.44	2.37	0.903			
	35	3.54	3.51	3.27	3.68	1.41	NS	*	NS
	70	2.96	3.00	2.76	3.03	0.894			
Monocytes, g/L	0	2.29	1.76	2.41	1.72	0.715			
	35	1.75	1.53	1.61	1.39	0.667	NS	NS	NS
	70	2.18	1.20	1.77	1.94	0.766			
Eosinophils, g/L	0	0.052	0.044	0.036	0.042	0.019			
	35	0.084	0.150	0.112	0.120	0.079	NS	***	NS
	70	0.056	0.098	0.065	0.092	0.051			
Basophils, g/L	0	0.257	0.499	0.228	0.370	0.396			
	35	0.170	0.258	0.074	0.132	0.154	NS	NS	NS
	70	0.239	0.221	0.199	0.267	0.207			
Not significant (NS). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$									

Figures

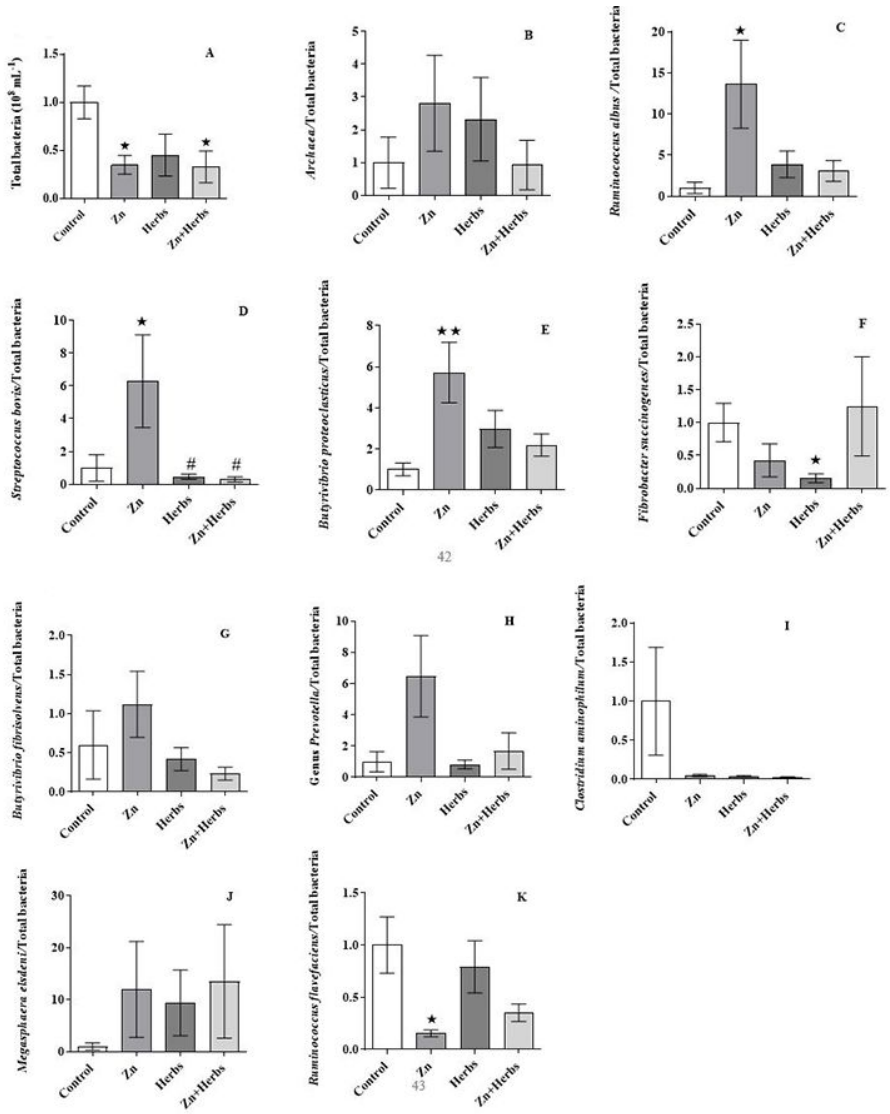

Figure 1

The effect of different diets (i.e., Control, Zn, Herbs and Herbs+Zn) on the relative 16S rRNA gene copy abundance (expressed relative to the total bacterial gene copy abundance of the Control) of the ruminal bacteria population (A) Total bacteria, (B) Archaea, (C) R. albus, (D) S. bovis, (E) B. proteoclasticus, (F) F. succinogenes, (G) B. fibrisolvens, (H) genus Prevotella, (I) C. aminophilum, (J) M. elsdeni and (K) R. flavefaciens. *P < 0.05, ** P < 0.01 differences from Control; # P < 0.05 differences from Zn.

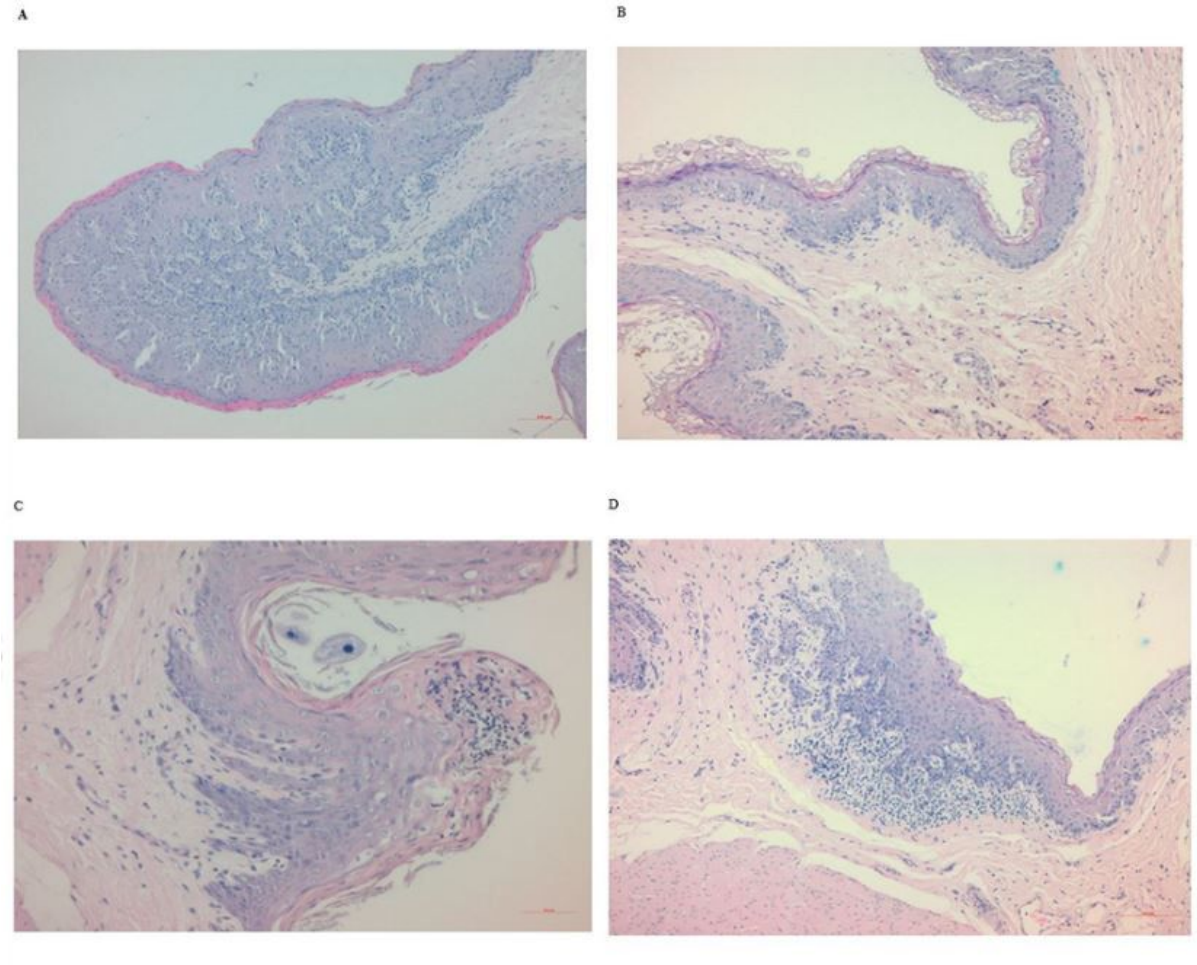

Figure 2

Histology of ruminal papillae of lambs fed different diets (A) Control, (B) Zn, (C) Herbs, and (D) Herbs+Zn stained by the hematoxylin-eosin method using the 10 × objectives.