BMC Veterinary Research

RESEARCH

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Efficacy of zinc nanoparticle supplementation on ruminal environment in lambs

Daniel Petrič[1], Klára Mikulová[1,2], Alexandra Bombárová[1,2], Dominika Batťányi[1], Klaudia Čobanová[1], Pavel Kopel[3], Anna Łukomska[4], Piotr Pawlak[5], Pola Sidoruk[6], Szymon Kotwica[6], Adam Cieslak[6*] and Zora Váradyová[1*]

Abstract

Background Zinc nanoparticles (NPs) are characterized by high bioavailability, small size, and high absorbability. The purpose of this experiment was to determine the effect of Zn-NP feed supplementation on ruminal fermentation, microbiota, and histopathology in lambs. In vitro (24 h), short-term (STE, 28 d), and long-term (LTE, 70 d) experiments were performed. The lambs in STE were fed a basal diet (BD) composed of 350 g/d ground barley and 700 g/d meadow hay (Control), BD enriched with ZnO-NPs (80 mg Zn/kg of diet, ZnO-NPs), and BD enriched with Zn phosphate-based NPs (80 mg Zn/kg of diet, ZnP-NP). The in vitro gas production technique was used in incubated rumen fluid from STE. The lambs in LTE were fed BD (Control), BD enriched with ZnO-NPs (40 mg Zn/kg of diet, ZnO-NP40), BD enriched with ZnO-NPs (80 mg Zn/kg of diet, ZnO-NP80) and BD enriched with ZnO (80 mg Zn/kg of diet, ZnO-80).

Results After 24 h of incubation, dry matter digestibility was higher for ZnO-NP and ZnP-NP substrates than the control in an in vitro experiment ($P < 0.001$). The total bacterial population in the STE was lower ($P < 0.001$) in the ZnP-NP group than in the control and ZnO-NP groups, but the protozoan populations were not significantly different. The ammonia-N concentration in LTE was lowest in the ZnO-NP80 group ($P = 0.002$), but the activities of carboxymethyl cellulase ($P < 0.001$) and xylanase ($P = 0.002$) were higher in the ZnO-NP40, ZnO-NP80, and ZnO-80 groups than in the control group. Morphological observation after STE and LTE revealed histological changes (e.g. inflammation of the epithelium or edema of the connective tissue) in the rumen of lambs.

Conclusion Zn-NP supplementation up to 70 d improved feed-use efficiency and influenced ammonia-N concentration and activities of hydrolases in the rumen. The active ruminal fermentation affected the health of the ruminal papillae and epithelium in the lambs, regardless of the application's form, dose, or duration. However, by affecting rumen microbial fermentation, Zn-NPs could alter fermentation patterns, thereby increasing the capacity of host rumen epithelial cells to transport short-chain fatty acids.

Keywords Ruminal microorganisms, Zinc nanoparticles, Fermentation, Ruminal histology

*Correspondence:
Adam Cieslak
adam.cieslak@up.poznan.pl
Zora Váradyová
varadyz@saske.sk
Full list of author information is available at the end of the article

Background

Zinc (Zn) plays catalytic, structural, and regulatory roles for enzymes, proteins, and transcription factors and improves immunological functions in ruminants [1, 2]. Zn in the diet of ruminants is usually supplemented as inorganic (e.g. ZnO and $ZnSO_4$) or organic (e.g. complexes of Zn and amino acids) substances. The requirements and recommendations for the content of dietary Zn for ruminants are between 40 and 130 mg/kg dry weight of the complete diet [3], but the bioavailability of Zn depends on the chemical form, content, and interaction with other dietary components [4, 5]. The use of organic sources of Zn in the diet of ruminants improves mineral supply due to the higher bioavailability and lower interference with other minerals because the binding of organic ligands from the organic form of trace elements should be more resistant to interactions in the digestive tract [6–8]. The nutritional effects of Zn sources on ruminant performance could be affected by factors such as the physiological stage and type of the animal, amount of dietary Zn, purity of supplements, presence or absence of stressors, storage of Zn in the body, environment, and season [8, 9]. The trace element Zn can affect the ruminal microbiota by positively influencing ruminal fermentation by directly acting on the activities of microbial enzymes. However, too high a concentration of Zn (adding > 50 µg/mL Zn to in vitro incubation) tends to reduce microbial activity, leading to a sharp drop in ammonia concentrations [2].

Nanoparticle (NP) minerals, due to their smaller size and more accessible transport through the gastrointestinal tract, prolong the residence time of the minerals, thereby ensuring a more uniform distribution and improving absorption into mucosal tissues and cells [10]. In vitro results have indicated that the addition of 20–30 mg ZnO-NP/kg dry matter (DM) to the diet could reduce methane emissions and improve total antioxidant capacity, the production of microbial biomass, digestibility of DM, and the efficiency of ruminal fermentation [11, 12]. Similarly, positive effects of inorganic and organic forms of Zn-NPs on ruminal fermentation, the digestibility of nutrients, antioxidant capacity, growth performance, and immunomodulatory and antibacterial effects have been described in ruminants [13–16]. The use of ZnO-NPs (28 mg Zn/kg DM) can also increase the ferric-reducing antioxidant power in the rumen and blood and decrease the level of blood urea-N in sheep [17]. The majority of studies support the beneficial effects of Zn nanoparticles on animal health [15, 16]. Only some studies have found cytotoxicity and histopathological changes after the administration of Zn-NPs [18, 19].

Currently, there is an urgent need to take advantage of advances in molecular chemistry, such as encapsulation techniques, to avoid the degradation of various additives in the rumen (e.g., trace elements, phytochemicals) and to use their nanostructures to increase the biological activity and availability of main substances that are less soluble in water. The higher antimicrobial activity of nanoparticles is related to their size in the subcellular size range. This allows the penetration of the nanoparticle into the microbial cells and leads to increased activity. Due to their small size and high surface-to-volume ratio, Zn-NPs are characterized by high bioavailability, resulting in their high absorbability and surface reactivity.

Our recent study showed that the ability of Zn dietary supplements (70 mg/kg diet) to affect ruminal microbial fermentation in vitro was not confirmed in vivo in lambs [20]. The rumen is covered by a stratified epithelium consisting of leaf-like papillae that allow the selective uptake of nutrients generated by intraruminal microbial fermentation [21]. These nutrients come mostly from the ruminal fermentation of dietary carbohydrates and are absorbed through the rumen epithelium. Therefore, we hypothesized that different zinc NPs (i.e., ZnO and ZnP) and doses would affect the rumen environment during in vitro and short- and long-term experiments in lambs. The purpose of this experiment was to determine the effect of Zn-NP feed supplementation on rumen fermentation, microbiota, and histopathology in lambs. Our objectives were to determine (1) the 24-h in vitro effect of Zn-NPs on parameters of ruminal fermentation and the protozoan population and (2) the short-term (28 d) and long-term (70 d) effects of Zn-NP supplementation on ruminal fermentation, the microbiota and histopathology in lambs.

Results

Twenty-four-hour in vitro experiment

The in vitro dry matter digestibility (IVDMD) was significantly higher in the ZnO-NP and ZnP-NP groups than in the control ($P < 0.001$, Table 1). The other parameters (pH, ammonia-N concentration, total gas production, methane concentration, and the concentrations of short-chain fatty acids (SCFAs)) were not significantly affected by the various Zn-NPs ($P > 0.05$). The total number of ciliates was not affected ($P > 0.05$).

Short-term effect on ruminal fermentation and the microbiota in lambs

Ammonia-N and total SCFA concentrations and the molar proportions of individual SCFAs did not differ significantly in the treated lambs ($P > 0.05$, Table 2). pH varied numerically between the groups but did not differ significantly. The total bacterial population was affected ($P < 0.001$) and was significantly lower in the ZnP-NP group than in the control and ZnO-NP groups. The

Table 1 Parameters of in vitro ruminal fermentation (mean ± SEM, $n = 9$)

Parameter	Control	ZnO-NPs	ZnP-NPs	*P*
pH	7.19 ± 0.04	7.19 ± 0.03	7.19 ± 0.03	0.990
IVDMD (g/kg DM)	451 ± 10.1[a]	546 ± 10.3[b]	573 ± 6.47[b]	0.001
Ammonia-N (mg/L)	113 ± 6.94	113 ± 10.5	120 ± 10.3	0.842
Total gas production (mL/g DM)	184 ± 6.48	184 ± 6.48	180 ± 6.67	0.858
Methane (mmoL)	2.61 ± 0.28	2.20 ± 0.22	2.19 ± 0.19	0.372
Total SCFAs (mM/L)	33.5 ± 1.46	32.1 ± 0.72	32.9 ± 0.99	0.667
Acetate (mol%)	68.6 ± 0.97	69.2 ± 1.06	68.0 ± 0.99	0.700
Propionate (mol%)	15.5 ± 0.52	15.3 ± 0.60	15.1 ± 0.51	0.904
n-Butyrate (mol%)	12.5 ± 0.26	12.2 ± 0.31	12.3 ± 0.29	0.799
iso-Butyrate (mol%)	0.88 ± 0.11	0.89 ± 0.13	0.82 ± 0.08	0.855
n-Valerate (mol%)	1.21 ± 0.09	1.21 ± 0.07	1.10 ± 0.06	0.526
iso-Valerate (mol%)	1.58 ± 0.15	1.62 ± 0.17	1.52 ± 0.14	0.899
n-Caproate (mol%)	0.15 ± 0.02	0.14 ± 0.01	0.12 ± 0.02	0.320
Acetate:propionate	4.47 ± 0.22	4.58 ± 0.27	4.62 ± 0.21	0.899
Total number of protozoa (10^3/mL)	6.81 ± 3.44	6.48 ± 3.43	6.24 ± 3.84	0.285

SEM standard error of the mean, *NPs* nanoparticles, *IVDMD* in vitro dry matter digestibility, *SCFAs* short-chain fatty acids

a,b: different letters within a row indicate significant differences at $P < 0.05$

Table 2 Effect of STE on ruminal fermentation and the microbiota (mean ± SD, $n = 9$)

Parameter	Control	ZnO-NPs	ZnP-NPs	*P*
pH	6.82 ± 0.27	6.90 ± 0.06	6.71 ± 0.21	0.540
Ammonia-N (mg/L)	92.0 ± 21.2	68.4 ± 15.5	61.4 ± 10.1	0.130
Total SCFAs (mM/L)	56.9 ± 16.5	40.9 ± 4.28	53.7 ± 10.1	0.272
Acetate (mol%)	72.3 ± 2.79	70.4 ± 0.20	69.3 ± 3.14	0.374
Propionate (mol%)	13.3 ± 1.09	14.8 ± 1.34	16.8 ± 4.27	0.346
n-Butyrate (mol%)	11.6 ± 0.74	11.4 ± 1.01	11.0 ± 1.72	0.808
iso-Butyrate (mol%)	0.78 ± 0.35	1.08 ± 0.29	0.85 ± 0.23	0.471
n-Valerate (mol%)	0.63 ± 0.12	0.67 ± 0.08	0.81 ± 0.28	0.465
iso-Valerate (mol%)	1.11 ± 0.50	1.32 ± 0.25	1.01 ± 0.14	0.545
Caproate (mol%)	0.25 ± 0.05	0.22 ± 0.05	0.30 ± 0.06	0.240
Acetate:propionate	5.46 ± 0.69	4.77 ± 0.43	4.32 ± 1.16	0.301
Total bacteria (10^8/mL)	2.69 ± 0.23[b]	2.63 ± 0.45[b]	2.35 ± 0.34[a]	0.001
Archaea (10^7/mL)	7.75 ± 0.20	7.66 ± 0.24	7.93 ± 2.38	0.867
Methanobacteriales (10^7/mL)	2.76 ± 0.50	2.67 ± 0.51	2.77 ± 0.52	0.595
Methanomicrobiales (10^7/mL)	2.67 ± 0.53	2.77 ± 0.52	2.78 ± 0.53	0.562
Total protozoa (10^5/g wRC)	29.0 ± 2.01	27.4 ± 1.67	27.0 ± 1.96	0.060
Holotricha (10^3/g wRC)	0.57 ± 0.21	0.74 ± 0.34	0.61 ± 0.27	0.352
Entodiniomorpha (10^3/g wRC)	28.2 ± 3.42	26.8 ± 7.10	26.0 ± 4.45	0.457
Specific enzymatic activities of the ruminal microorganisms (µcat/g protein)				
α-Amylase	1.66 ± 0.80	1.23 ± 0.63	1.86 ± 0.57	0.126
Carboxymethyl cellulase	1.13 ± 0.43	1.45 ± 0.99	1.02 ± 0.55	0.302
Xylanase	179 ± 24.2	189 ± 26.2	202 ± 35.8	0.144

STE short-term experiment, *NPs* nanoparticles, *wRC* count per gram wet ruminal content, *SCFAs* short-chain fatty acids. Different letters within a row indicate significant differences at $P < 0.05$

protozoan population did not differ significantly between the groups ($P > 0.05$). The specific enzymatic activities of α-amylase, carboxymethyl cellulase (CM-cellulase), and xylanase of the ruminal microorganisms were not affected ($P > 0.05$).

Short-term effect on ruminal histology

Medium-sized warts, connective-tissue edema, and organisms with the morphology of *Balantidium coli* were observed ($P = 0.032$, Table 3). The epithelial keratinocyte layer differed mainly between the ZnO-NP and ZnP-NP groups ($P = 0.008$). The connective tissue of the papillae was inflamed in all sheep. Damage to homogeneous papillae and erosion of the hyperplastic stratum corneum did not differ significantly between the groups ($P > 0.05$).

Long-term effect on ruminal fermentation and the microbiota

The concentration of ammonia-N was significantly lower in the ZnO-NP80 group than the other groups ($P = 0.002$), and the molar proportion of *n*-valerate was significantly lower in the ZnO-NP80 group than the control group ($P = 0.015$, Table 4). The molar proportion of caproate differed significantly between the ZnO-NP40 and ZnO-80 groups ($P = 0.030$). The activities of CM-cellulase ($P < 0.001$) and xylanase ($P = 0.002$) were significantly higher in all three experimental groups than in the control group. The protozoan population did not differ significantly between the groups ($P > 0.05$). The populations of *Butyrivibrio proteoclasticus*, *B. fibrisolvens*, *Fibrobacter succinogenes*, *Prevotella* spp., *Ruminococcus*

Table 3 Effect of STE on the histopathology of ruminal tissues (mean ± SD, $n = 9$)

Parameter (%)	Control	ZnO-NPs	ZnP-NPs	*P*
Medium-sized warts	89 ± 33.3	100 ± 0.0	100 ± 0.0	0.032
Homogenous papillae	89 ± 33.3	33 ± 50.0	89 ± 33.3	0.282
Inflammation of connective tissue of papillae	100 ± 0.0	100 ± 0.0	100 ± 0.0	–
Connective-tissue edema	100 ± 0.0	89 ± 33.3	100 ± 0.0	0.032
Epithelium (keratinocyte layer)	89 ± 33.3^{ab}	33 ± 50.0^{a}	100 ± 0.0^{b}	0.008
Erosion of hyperplastic stratum corneum	33 ± 50.0	0 ± 0.0	33 ± 50.0	0.157
Other (*Balantidium coli*)	100 ± 0.0	89 ± 33.3	100 ± 0.0	0.032

STE short-term experiment, *NPs* nanoparticles, *SD* standard deviation

a,b: different letters within a row indicate significant differences at $P < 0.05$

Table 4 Effect of LTE on ruminal fermentation and microbiota in lambs (mean ± SD, $n = 7$)

Parameter	Control	ZnO-NP40	ZnO-NP80	ZnO-80	*P*
pH	6.41 ± 0.34	6.50 ± 0.44	6.37 ± 0.29	6.33 ± 0.22	0.124
Ammonia-N (mg/L)	70.1 ± 22.0^{b}	54.5 ± 28.7^{b}	27.4 ± 11.0^{a}	69.1 ± 29.5^{b}	0.002
Total SCFAs (mM/L)	61.1 ± 11.3	50.1 ± 14.5	52.3 ± 6.60	51.4 ± 4.49	0.204
Acetate (mol%)	67.6 ± 3.19	67.8 ± 4.24	68.6 ± 2.09	68.8 ± 2.47	0.875
Propionate (mol%)	17.0 ± 1.44	17.9 ± 2.32	17.4 ± 1.78	17.4 ± 2.34	0.889
n-Butyrate (mol%)	13.2 ± 1.62	12.1 ± 2.64	12.2 ± 1.48	11.8 ± 0.618	0.534
iso-Butyrate (mol%)	0.52 ± 0.41	0.54 ± 0.23	0.41 ± 0.14	0.31 ± 0.10	0.376
n-Valerate (mol%)	1.22 ± 0.16^{b}	1.11 ± 0.11^{ab}	0.92 ± 0.12^{a}	1.06 ± 0.23^{ab}	0.015
iso-Valerate (mol%)	0.39 ± 0.26	0.57 ± 0.17	0.39 ± 0.27	0.51 ± 0.34	0.491
Caproate (mol%)	0.11 ± 0.10^{ab}	0.06 ± 0.06^{a}	0.10 ± 0.04^{ab}	0.18 ± 0.04^{b}	0.030
Acetate:propionate	4.01 ± 0.46	3.86 ± 0.63	3.98 ± 0.51	4.02 ± 0.61	0.954
Total protozoa (10^5/g wRC)	20.9 ± 3.56	17.5 ± 5.72	20.7 ± 5.19	17.9 ± 5.82	0.175
Specific enzymatic activities of the ruminal microorganisms (μcat/g protein)					
α-Amylase	1.10 ± 0.85	1.75 ± 1.48	1.24 ± 0.94	0.92 ± 0.56	0.210
Carboxymethyl cellulase	0.42 ± 0.22^{a}	1.06 ± 0.37^{b}	0.83 ± 0.20^{b}	0.80 ± 0.32^{b}	0.001
Xylanase	37.2 ± 8.90^{a}	67.4 ± 17.2^{b}	64.8 ± 15.6^{b}	58.8 ± 26.3^{b}	0.002

LTE long-term experiment, *SCFAs* short-chain fatty acids, *wRC* count per gram wet ruminal content, *NPs* nanoparticles, *SD* standard deviation

a,b: different letters within a row indicate significant differences at $P < 0.05$

albus, *R. flavefaciens*, *Streptococcus bovis* and *Total methanogens* were not affected ($P > 0.05$, Fig. 1).

Effects of long-term supplementation of Zn on ruminal histology

The size of the ruminal papillae varied in all sheep (i.e. short and thick, long and thin, and medium length and width, Table 5). The epithelial keratinocyte layer differed mainly between the ZnO-NP40 and ZnO-NP80 groups ($P = 0.038$), but connective-tissue edema occurred primarily in the control group ($P = 0.009$). The flat and thin or rough layer of desquamating and ballooning keratinocytes particularly characterized the histopathological changes to the ruminal epithelium (i.e., epithelium and lamina propria inflammation with infiltrates of inflammatory cells, mainly lymphocytes). Organisms with *B. coli* morphology were present in almost all groups. The histological changes are shown in Fig. 2a and b.

Discussion

Our previous in vitro results indicated that the fermentation of 25 mg of organic Zn in the ruminal fluid collected from lambs fed for 70 d with a diet containing Zn at a dose of 70 mg/kg DM decreased gas production and IVDMD [20]. The present study using a technique of 24-h in vitro gas production (IVGPT), however, did not detect any adverse effects of the Zn-NPs on ruminal fermentation or the protozoan population. Instead, microbial populations and parameters of ruminant fermentation

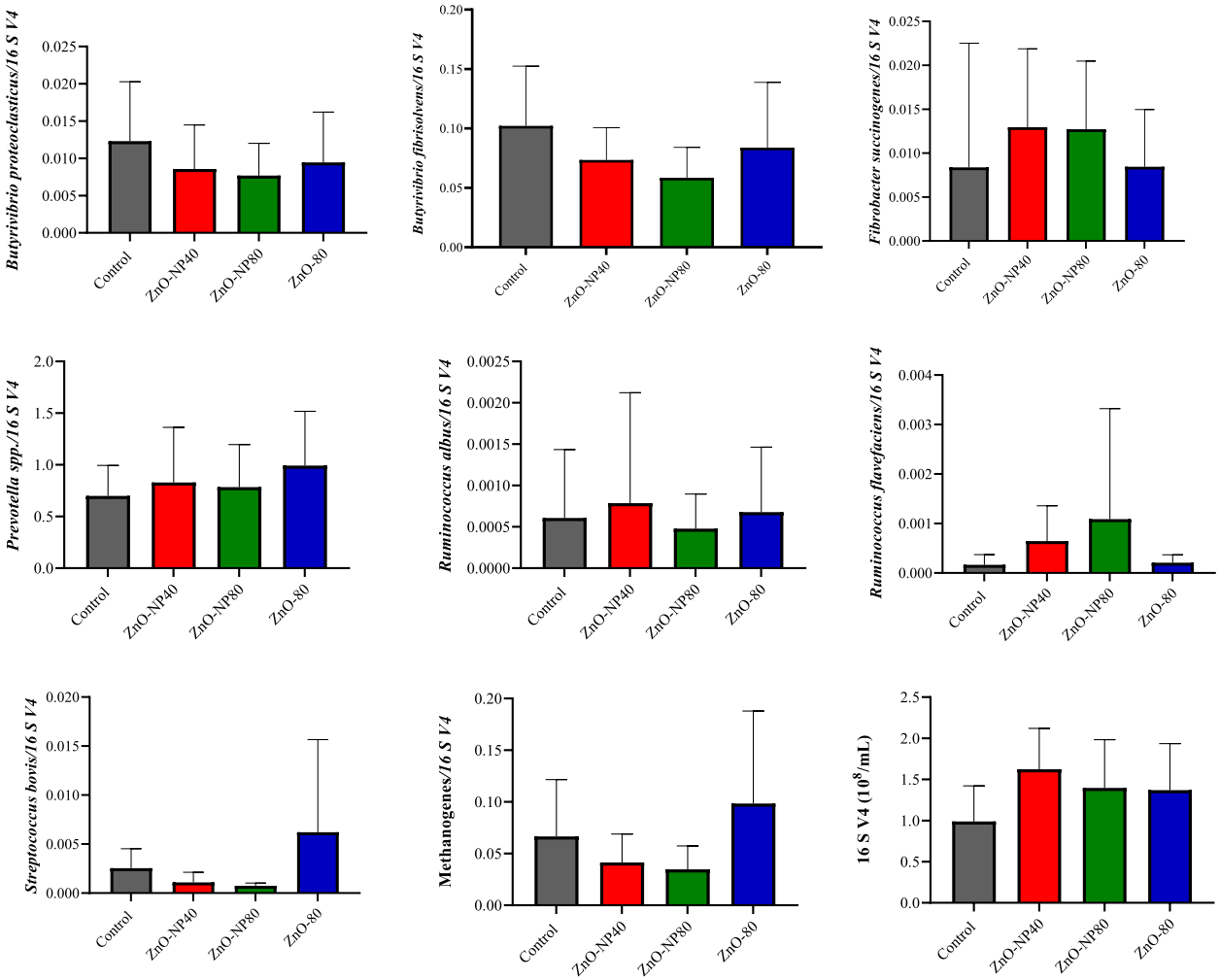

Fig. 1 Effect of the control and experimental groups on the relative abundance of the 16S rRNA gene (expressed relative to the total abundance of bacterial genes) of the ruminal bacterial population for *Butyrivibrio proteoclasticus*, *Butyrivibrio fibrisolvens*, *Fibrobacter succinogenes*, *Prevotella* spp., *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Streptococcus bovis*, and *Total methanogens*. Data are described as specific gene copy number per 16 s rRNA gene copy number ± SEM ($P > 0.05$)

Table 5 Effect of LTE on histopathology of ruminal tissues (means ± SD, $n = 7$)

Histological change (%)	Control	ZnO-NP40	ZnO-NP80	ZnO-80	*P*
Size of ruminal papillae	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	-
Epithelium (keratinocyte layer)	57 ± 53.5^{ab}	100 ± 0.0^{b}	29 ± 48.8^{a}	43 ± 53.5^{ab}	0.038
Inflammation of lamina propria	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	-
Epithelial inflammation	43 ± 53.5	71 ± 48.8	86 ± 37.8	86 ± 37.8	0.264
Connective-tissue edema	100 ± 0.0^{b}	86 ± 37.8^{ab}	29 ± 48.8^{a}	43 ± 53.5^{a}	0.009
Other (*Balantidium coli*)	86 ± 37.8	100 ± 0.0	100 ± 0.0	100 ± 0.0	0.410

LTE long-term experiment, *NPs* nanoparticles, *SD* standard deviation. Different letters within a row indicate significant differences at $P < 0.05$

could probably be beneficially modified by Zn-NP supplementation [22], and therefore IVDMD was increased. Our findings are similar to those observed with diets containing 20–60 mg/kg ZnO-NPs using 24-h IVGPT [23]. However, inconsistent with the present study, some previous studies reported that a dose of 20–80 mg/kg of ZnO-NP DM was sufficient to improve ruminal fermentation and reduce the concentration of methane released

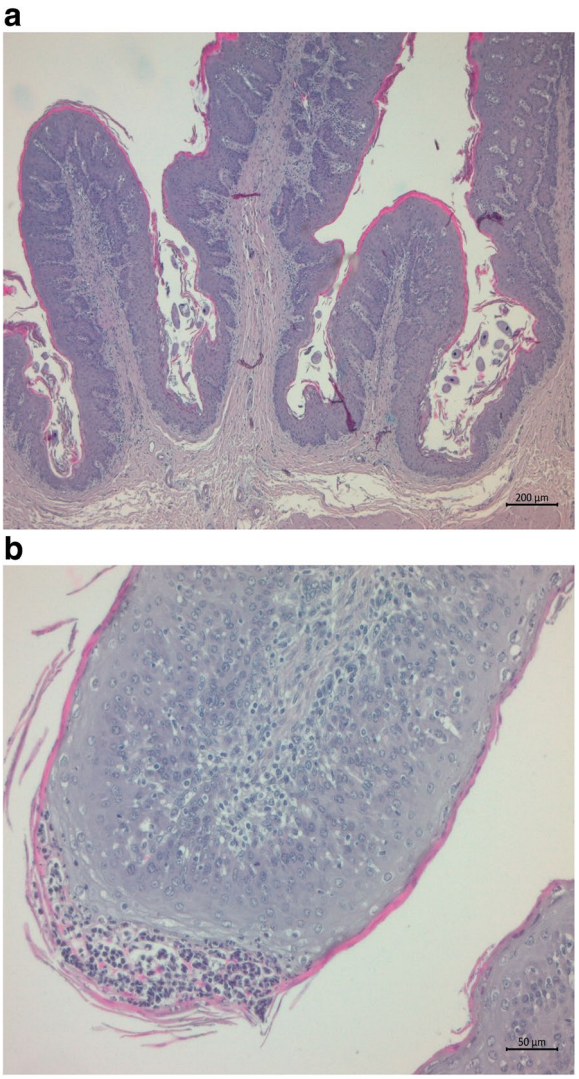

Fig. 2 **a** Histological changes to ruminal tissue in LTE: Ruminal papillae with the desquamation of keratinocytes. **b** Histological changes to ruminal tissue in LTE: Focal aggregates of inflammatory cells with a predominance of neutrophils in the epithelium of the ruminal papilla

in vitro [23, 24]. In vitro measurements after 24 h have indicated that methane concentration and total protozoan population tend to decrease more in incubations with Zn-NPs than with other sources of Zn (e.g. ZnO) [23], consistent with our results. The toxicity of the ZnO-NPs caused by dissolved metal ions on ciliated ruminal protozoa, however, probably decreases after 24 h of exposure due to protozoan adaptation [25]. The microbial population and the concentration of fermentation gases, however, probably decrease greatly after 72 h of in vitro incubation with higher doses of ZnO-NPs (500–1000 mg/kg) [26]. The dietary substrates containing ZnO-NPs and ZnP-NPs at doses of 80 mg/kg DM in our experiment, however, had the potential to increase substrate IVDMD. Other studies have also described a positive effect on the increase in IVDMD by supplementing diets with ZnO-NPs [12, 27], consistent with our results. Supplementation with ZnO-NPs at the dose of 30–40 mg Zn/kg DM increased DM digestibility in the rumen [28] as ZnO-NPs have better bioavailability, enhance microbial population and increase substrate breakdown thereby improving dry matter digestibility of feedstuffs [29]. The dose of 90–180 mg/kg DM, however, gradually decreased IVDMD, probably due to the antibacterial activity of the ZnO-NPs and the suppression of the growth of the ruminal microbial population [12]. The effect on digestibility and the microbiota clearly depended mainly on the dose of Zn-NPs.

In vitro measurements can predict the parameters of ruminal fermentation with reasonable accuracy, but we needed to identify the effect of the Zn-NPs in the rumen in vivo. STE with Zn-NP supplementation did not affect the parameters of fermentation or specific microbial enzymatic activity. The total bacterial population decreased, and the protozoan population tended to decrease mainly with dietary ZnP-NP supplementation, despite the unchanged fermentation profile. Likewise, in an in vivo study, ZnP-NPs may directly affect bacterial activity in the rumen during short-term supplementation while the group with ZnO-NPs showed no effect on bacterial population. ZnP-NPs could probably alter microbial populations due to adaptation to a diet without adverse effects on fermentation [23]. Moreover, in the case of protozoa, both ZnP-NPs and ZnO-NPs showed no effect on their population. ZnP-NP supplementation can have a short-term effect on the growth of bacteria in the rumen, with subsequent impacts on the formation of microbial proteins and the use of energy. The production of proteins by ruminal microbes, however, is probably inefficient, mostly due to maintenance functions, decreasing bacterial population [12], and the accumulation of reserve saccharides by protozoa [30].

The optimal level of ammonia-N in the rumen (20–100 mg/L) [31] was not exceeded in LTE. Ammonia-N is normally the most abundant source of N required for microbial growth, and its lower concentration in the rumen may be due to the higher consumption of ammonia-N by microorganisms, the presence of low-level rumen degradable protein, and optimum pH in the rumen [2]. The decrease in ammonia-N concentration in the ZnO-NP80 group was not correlated with the significant changes to the ruminal microbiota, although its decrease may have been due to the higher use of ammonia-N by the microbial population. If ruminal microorganisms have access to a readily available source of

energy, they increase their synthesis of proteins using amino acids as a microbial source of energy [32]. The lack of significant differences in the microbiotas in the experimental groups may have been due to the different forms of Zn used than in our previous study [20]. The relatively high standard deviations of the means of the bacteria in the experimental groups indicated a potentially different effect between lambs, suggesting that different forms and doses could also have different antibacterial and antimethanogenic effects and may promote fermentation in the rumen to reduce the concentration of methane released in the long term [24] (e.g. by Total methanogens, Fig. 1). The LTE diets containing ZnO-NPs and ZnO did not significantly affect total SCFA concentration or the microbiotas in the rumens of the lambs. Similarly, the metabolism of dietary saccharides was probably unaffected; the concentrations of individual SCFAs changed only slightly (a slight effect on *n*-valerate and caproate). Zn in the diet, however, can substantially affect ruminal fermentation [33, 34], although probably weakly at low doses (20–70 mg Zn/kg diet) [35, 36]. Higher doses (250–1100 mg Zn/kg diet) can affect the population of ruminal protozoa and protein degradation [37]. ZnO supplementation at a dose of 10–50 mg Zn/kg DM increased the concentration of total SCFAs, despite the likely low solubility of ZnO in the rumen [38]. Only a small part of the Zn supplement is probably solubilized during fermentation; ZnO is poorly assimilated by ruminal bacteria, and ruminal protozoa preferably assimilate highly soluble Zn [38], which may indicate a shift in saccharide fermentation by the protozoal population at the expense of bacterial fermenters [39], which our experiment did not detect.

Finally, supplementation with ZnO-NPs (30–40 mg Zn/kg DM) in the pre- and post-partum periods in sheep can increase the total SCFA concentration in the rumen [28]. This finding may also indicate an improvement in the activities of digestive enzymes, especially protease, amylase, and lipase, leading to higher starch digestibility and thus higher SCFA concentrations [40]. Too much or too little Zn in the diet of ruminants, however, probably has the opposite effect [41, 42]. LTE in our study did not affect the ruminal microbiota, unlike STE. This finding probably indicates a gradual adaptation of the microbiota to the zinc diets during LTE [43]. Ruminal microbiotas, however, have different sensitivities to Zn, and the currently recommended levels of Zn intake are defined to meet the needs of the animal, not the requirements of the ruminal microbiota [23, 38]. Our results suggest that the effects of Zn on ruminal fermentation and the microbiota also strongly depend on the duration of Zn supplementation. The different forms of Zn applied, such as NPs or ZnO, had very similar effects during LTE. The lack of an apparent inhibition or improvement of the parameters of ruminal fermentation, though, suggests that the ruminal microbiota may have too low a requirement for Zn supplementation [38]. The increased specific enzymatic activities of the ruminal microorganisms, especially cellulase and xylanase, however, indicated that Zn is incorporated into enzymes throughout the body and is crucial for most metabolic processes in ruminants [44]. Zn is therefore involved in a wide range of physiological processes, such as the digestion of nutrients, which can be affected by long-term Zn supplementation. The ruminal microbiotas of the Zn groups in our experiment were probably more than the control associated with cellulase and xylanase activities that accelerated biodegradation during the ruminal processing of substrates [45]. Zn likely supports the efficient digestion of complex substrates in the rumen, which requires the coordinated action of many enzymes that can act individually and synergistically, or individual enzymes could assemble into multienzyme complexes [46].

The ruminal papillae in STE were homogeneous and associated with inflammation of the connective tissue in almost all lambs. The keratinocyte layer of the epithelium was badly damaged in the ZnP-NP group, which may have negatively affected the epithelium because the outer layer of keratinized cells of the ruminal epithelium is an absorption barrier for the transport of molecules from the rumen to the blood [47]. Almost all lambs in STE had connective-tissue edema, which in LTE was mainly in the control group. The ruminal papillae in LTE were short and thick, long and thin, medium long, and wide, but inflammation of the lamina propria was present in all lambs. Other damage (e.g. inflammation and connective-tissue edema) was probably caused by dystrophic epithelial changes that led to cellular degeneration and the infiltration of leukocytes. These lesions can affect absorption capacity and stimulate inflammation and secondary ruminal infection by the resident microbial population [48]. Butyrate stimulates the development of ruminal papillae [49], but the molar proportion of butyrate was not affected in our experiments. The growth and development of the ruminal papillae therefore probably depended mainly on the type of feed consumed. However, the end products of ruminal fermentation such as butyrate production rather than the nature of the feed, stimulate the development of ruminal papillae [50]. The inconsistent results for valerate in LTE may therefore indicate increased surface area and epithelial thickness associated with an increased absorptive capacity for valerate with diffuse uptake [51]. SCFAs in the rumen are

absorbed through the ruminal epithelium, and the rate of absorption is primarily influenced by their concentration, the surface area of the papillae in the rumen, and the availability of transport proteins [52, 53]. SCFAs, as products of ruminal fermentation, generally induce morphofunctional changes to the ruminal papillae [54] and probably moderate the effect on the keratinocyte layer of the epithelium and connective-tissue edema in some lambs, but further studies are needed.

Conclusion

Our research pointed out the potential of the Zn-NP supplementation to improve feed-use efficiency in the LTE up to 70 d. The ability of long-term Zn-NP supplementation to affect ruminal fermentation parameters was supported by its effect on ammonia-N concentration and microbial hydrolase activity. The active microbial fermentation in the rumen was likely to affect the health of the ruminal papillae and epithelium in the lambs, regardless of the application's form, dose, or duration. However, by affecting rumen microbial fermentation, Zn-NPs could alter fermentation patterns, thereby increasing the capacity of host rumen epithelial cells to transport SCFAs.

Methods

Ethical study

This study was conducted following the guidelines of the Declaration of Helsinki and national legislation in the Slovak Republic (G.R. 377/2012; Law 39/2007) for the care and use of research animals. The experimental protocol was approved by the Ethical Committee of the Institute of Animal Physiology, Centre of Biosciences of the Slovak Academy of Sciences on 07 March 2023 (protocol code 1046/2023).

Animals, diets, and design of STE

Twenty-seven lambs (5–6-month-old rams, Improved Valachian) were housed in separate pens for 30 days for acclimatization with free access to water. The number of animals used in the experiment was assigned according to VICH GL13 guidelines proposed by the European Medicines Agency. The lambs were obtained from a commercial farm (PD Ružín–Ružín farm, Kysak, Slovakia) and were housed at the Research Centre of the Institute of Animal Physiology of Centre of Biosciences of Slovak Academy of Sciences. No criteria for inclusion and exclusion of animals were used during the experiment. The confounders were not controlled. After acclimatization the lambs with body weights of 21.2 ± 1.1 kg (mean ± standard error of the mean) were fed an experimental diet in three groups ($n = 9$/group): (a) a basal diet (BD) composed of 350 g/d ground barley and 700 g/d meadow hay, (b) BD enriched with ZnO-NPs (80 mg Zn/kg of diet, ZnO-NPs) and (c) BD enriched with Zn phosphate-based NPs (80 mg Zn/kg of diet, ZnP-NP). The ZnO-NPs were a commercial product (zinc oxide nanopowder, < 100 nm particle size, Sigma-Aldrich, Saint-Louis, USA). The ZnP-NPs were chemically synthesized (Department of Inorganic Chemistry, Palacky University, Olomouc, Czech Republic) following the published method described in detail [55]. ZnP-NPs were characterized (particle size and shape, structural analysis) by scanning electron microscopy, transmission electron microscopy, and X-ray diffraction (unpublished data). The study design is experimental and includes compared groups of animals including control groups. For the treatment groups, aliquots of zinc supplements were mixed directly with the feed concentrate (ground barley) for each meal to provide an additional zinc diet. The experimental period was 28 d and the lambs were euthanized following the rules of the European Commission (Council Regulation 1099/2009) [56]. Twenty-seven lambs were killed over three consecutive days and the rumen fluid for in vitro experiments was pooled. All lambs with an average body weight of 24 kg were euthanized by using an overdose of 96 mg/kg of pentobarbital (Dolethal, Vetoquinol, UK, Ltd.) on 28 d of the experiment (abattoir of the Centre of Biosciences of SAS, Institute of Animal Physiology, Košice, Slovakia, No. SK U 06018). Pentobarbital overdose had a negligible effect on the estimated parameters of the ruminal environment in lambs. The carcasses were sent to the Department of Pathological Anatomy and Pathological Physiology, University of Veterinary Medicine and Pharmacy in Košice in the Slovak Republic.

Twenty-four-hour in vitro experiment

The Zn-NPs were incubated in vitro in the ruminal fluid (RF) to assess their effect on the parameters of ruminal fermentation (pH, ammonia-N concentration, gas production, methane concentration, and SCFA concentrations). RF was collected from the ruminal contents of slaughtered lambs at the end of STE. RF was obtained before morning feeding, strained through four layers of gauze into thermal flasks, and immediately transported to the laboratory to exclude external factors that may affect the estimated parameters. RF was mixed at a 1:2 ratio with McDougall's buffer [57], and purged with CO_2. The RF inoculum was dispensed in volumes of 35 mL into serum bottles (120 mL) containing 0.25 g of substrate. Ground barley without (Control) or with Zn-NPs (80 mg Zn/kg DM, ZnO-NPs or ZnP-NPs) and meadow hay (350:700, w/w) were used as substrates for the in vitro experiment and fermented with buffered RF. ZnO-NPs (zinc oxide nanopowder, < 100 nm particle

size, Sigma-Aldrich, Saint-Louis, USA) and ZnP-NPs (Department of Inorganic Chemistry, Palacky University, Olomouc, Czech Republic) were used. The serum bottles with buffered ruminal fluid and substrate were filled with CO_2, closed with rubber stoppers and sealed with aluminum cups. Then the bottles were incubated in an incubator (Galaxy 170R, Eppendorf North America Inc., Hauppauge, NY) for 24 h at a temperature of 39 ˚C in an anaerobic condition with periodical mixing of the contents. The experimental design used the in vitro gas production technique as a relatively simple method to evaluate feedstuffs in ruminants. Three replicates (three incubation serum bottles) were prepared for each substrate (i.e., Control, ZnO-NPs, ZnP-NPs), and the experiment was conducted three times within three consecutive days ($n = 3 \times 3$). Three replicate bottles were also used for the blank (ruminal inoculum, no substrate).

Animals, diets, and design of LTE

Twenty-eight male lambs (4 months of age, Improved Valachian) were housed in common stalls for 30 days for acclimatization with free access to water. The number of animals used in the experiment was assigned according to VICH GL13 guidelines proposed by the European Medicines Agency. The lambs were obtained from a commercial farm (PD Oľšavica-Brutovce, Slovakia) and were housed at the Research Centre of the Institute of Animal Physiology of the Centre of Biosciences of Slovak Academy of Sciences. No criteria for inclusion and exclusion of animals were used during the experiment and the confounders were not controlled. After acclimatization the lambs (body weights of 20.19 ± 0.50 kg) were fed an experimental diet in four groups ($n = 7$/group): (a) BD composed of 350 g/d ground barley and 700 g/d meadow hay, (b) BD enriched with ZnO-NP (40 mg Zn/kg of diet, ZnO-NP40, SkySpring Nanomaterials, Inc., Houston, USA), (c) BD enriched with ZnO-NPs (80 mg Zn/kg of diet, ZnO-NP80, SkySpring Nanomaterials, Inc., Houston, USA), and (d) BD enriched with ZnO (80 mg Zn/kg of diet, ZnO-80, Sigma-Aldrich, Saint-Louis, USA). The experimental period of LTE was 70 d. The study design was experimental and included compared groups of animals including control groups. For the treatment groups, aliquots of zinc supplements were mixed directly with the feed concentrate (ground barley) for each meal to provide an additional zinc diet. All sheep with an average body weight of 35 kg were euthanized using an overdose of 140 mg/kg of pentobarbital (Dolethal, Vetoquinol, UK, Ltd.) at the end of the experiment at the abattoir as described in STE. Pentobarbital overdose had a negligible effect on the estimated parameters of the ruminal environment in lambs. The carcasses were sent to the Department of Pathological Anatomy and Pathological Physiology, University of Veterinary Medicine and Pharmacy in Košice in the Slovak Republic.

Measurements and chemical analysis

The dietary substrates were analyzed in triplicate using standard procedures [58]. The DM content was obtained by drying the samples at 105 °C for at least 24 h in an oven (method no. 930.15). The total ash content of the samples was determined by ashing overnight at 550 °C (method no. 942.05) in a muffle furnace. Nitrogen content (method no. 968.06) was determined using a FLASH 4000 analyzer (Thermo Fisher Scientific, Cambridge, UK). Crude-protein (CP) content was calculated by multiplying the total N content by 6.25 (method no. 990.03). The acid-detergent and neutral-detergent fiber contents were analyzed as described previously [59] using an ANKOM 2000 analyzer (ANKOM Technology, Macedon, USA) with heat-stable α-amylase. The chemical compositions of the dietary substrates are provided in Table 6.

pH was measured using a pH meter (InoLab pH Level 1, Weilheim, Germany). IVDMD was estimated as the difference in substrate weights before and after incubation [60]. The concentration of ammonia-N in

Table 6 Chemical composition of the dietary substrates (g/kg DM)

	STE				LTE				
Substrate	**MH**	**BG**	**ZnO-NPs**	**ZnP-NPs**	**MH**	**BG**	**ZnO-NP40**	**ZnO-NP80**	**ZnO-80**
DM (g/kg)	884	879	882	881	896	880	879	877	878
NDF	691	266	275	265	716	327	292	305	307
ADF	365	85	90	91	351	103	108	109	127
CP	44	130	124	125	41	112	114	113	106
N	10	21	20	20	7	18	18	18	17
Ash	43	38	39	41	52	29	34	34	37

STE short-term experiment, *LTE* long-term experiment, *MH* meadow hay, *BG* ground barley, *NPs* nanoparticles, *DM* dry matter, *NDF* neutral-detergent fiber, *ADF* acidic-detergent fiber, *CP* crude protein, *N* nitrogen. The aliquots of the Zn supplements were directly mixed with the BG for each meal to provide a Zn diet (i.e., ZnO-NPs, ZnP-NPs, ZnO-NP40, ZnO-NP80 and ZnO-80, respectively)

the ruminal fluid was determined using the phenol-hypochlorite method [61]. The volume of accumulated gas/pressure released by IVGPT after 24 h of in vitro fermentation was determined using a mechanical manometer fitted to a transducer (Premagas, Stará Turá, Slovak Republic) [62].

The SCFA and methane concentrations were analyzed using a Clarus 500 gas chromatograph (Perkin Elmer, Inc., Shelton, USA) [63]. The chromatograph was equipped with a flame ionization detection system for estimating the SCFA and methane concentrations. The SCFAs were separated using a stainless-steel packed column (2 m × 2 mm i.d.) with a phase composition of 10% Carbowax 20 M-TPA + 1% H_3PO_4 on a 100/120 Supelcoport support (Supelco, Bellefonte, USA). The methane concentration was analyzed using a 10% Squalane Chrom P mesh side 80/100 packed column, 2 m × 2 mm i.d. (Supelco, Bellefonte, USA), and a peak was observed at 0.33 min. The column oven temperature was programmed at 150 °C. Injector and detector temperatures were programmed at 230 °C. The rates of gas flow were 40 mL/min for hydrogen and 400 mL/min for air in both analyses. The average flow of nitrogen carrier gas was set at 36 *psi* for SCFA and 14 *psi* for methane.

Specific enzymatic activities

The specific enzymatic activities of the ruminal microorganisms were determined by preparing a cell-free homogenate from the ruminal content of the lambs [64]. Activity was expressed in units of specific catalytic activity (cat/g of protein). The activity of α-amylase was determined using 0.2% (w/v) maize starch (Merck KGaA, Darmstadt, Germany) resuspended in 0.05 M phosphate-citrate buffer. The activity of CM-cellulase was determined using 1% (w/v) carboxymethyl cellulose (Merck KGaA, Darmstadt, Germany). Xylanase activity was determined using 1% (w/v) Beechwood xylan (Merck KGaA, Darmstadt, Germany) resuspended in the same phosphate-citrate buffer. Enzymatic activities were determined by measuring the amount of reducing sugars released from cell-free samples of ruminal homogenate after 15 min at 39 °C.

Microbial analyses and quantification

Samples for counting ciliate protozoa (i.e., in vitro, STE, LTE) were fixed in equal volumes of 8% formaldehyde, and the protozoa were counted and identified microscopically [65]. In the STE experiment total bacteria, *Archaea*, *Methanobacteriales*, and *Methanomicrobiales* were quantified using fluorescence in situ hybridization [66]. In the LTE study, samples were isolated by PureLink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher) according to manufacturer protocol. The concentration of DNA was measured using Nanodrop 1C. Quantitative PCR was performed on Roche Light Cycler 480 II using standard curves for absolute quantification of specific taxa and total bacteria by 16S subunit gene amplification. The primers used are summarized in Table 7. Data are presented as a copy number of specific amplicon per total bacteria in the sample. Data are described as specific gene copy numbers per 16 s rRNA gene copy number ± SEM.

Table 7 The sequences of primers specific to the analyzed bacteria species

Species	Primer sequences	Reference
Ruminococcus flavefaciens	F – 5′ CGAACGGAGATAATTTGAGTTTACTTAGG 3′ R – 5′ CGGTCTCTGTATGTTATGAGGTATTACC 3′	[67]
Fibrobacter succinogenes	F – 5′ GTTCGGAATTACTGGGCGTAAA 3′ R –5′ CGCCTGCCCCTGAACTATC 3′	[68]
Streptococcus bovis	F – 5′TTCCTAGAGATAGGAAGTTTCTTCGG 3′ R – 5′ ATGATGGCAACTAACAATAGGGGT 3′	[69]
Butyrivibrio proteoclasticus	F – 5′TCCTAGTGTAGCGGTGAAATG 3′ R –5′TTAGCGACGGCACTGAATGCCTA 3′	[70]
Ruminococcus albus	F – 5′ CCCTAAAAGCAGTCTTAGTTCG 3′ R – 5′ CCTCCTTGCGGTTAGAACA 3′	[71]
Butyrivibrio fibrisolvens	F – 5′ ACACACCGCCCGTCACA 3′ R – 5′TCCTTACGGTTGGGTCACAGA 3′	[72]
Prevotella spp.	F – 5′ GAAGGTCCCCCACATTG 3′ R – 5′ CAATCGGAGTTCTTCGTG 3′	[69]
Total methanogens	F – 5′ GAGGAAGGAGTGGACGACGGTA 3′ R – 5′ ACGGGCGGTGTGTGCAAG 3′	[73]
16 S V4	F – 5′TATGGTAATTGTGTGNCAGCMGCCGCGGTAA 3′ R – 5′ AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT 3′	[74]

Histology

Histological examinations were performed on samples of fresh ruminal tissues washed in a phosphate buffer (0.1 M, pH 7.4), put in plastic containers, and fixed in a 10% buffered formalin solution as pieces of tissue spread on flat polystyrene [20]. The fixed material was processed using a series of reagents and embedded in Paraplast PLUS paraffin blocks (Leica, Buffalo Grove, USA), which were then cut using a rotary microtome into Sects. 3.5 µm thick. Slides with a paraffin section were automatically stained with hematoxylin and eosin (Varistain Gemini Thermo Scientific, Runcorn, UK). An Axio Lab. 1 microscope (Carl Zeiss, Jena, Germany) equipped with a Zeiss Axiocam ERc5s digital camera and Imager.M2 Axio (Carl Zeiss, Jena, Germany) was used for histological evaluation. Photographs were analyzed and recorded using ZEN 2.3 (blue edition) software (Carl Zeiss Microscopy GmbH, 2011).

Statistical analysis

The data were analyzed using one-way ANOVAs (GraphPad Prism 9.2.0 (332) 2021; GraphPad Software, Inc., San Diego, USA). Individual differences were determined using Tukey's multiple-comparison post hoc test and were considered to be significant at $P<0.05$. The microbial population data were evaluated using the nonparametric Kruskal–Wallis method.

Abbreviations

ADF	Acid detergent fiber
ANOVA	Analysis of variance
BD	Basal diet
CP	Crude protein
CM-cellulase	Carboxymethyl cellulose
DM	Dry matter
IVDMD	in vitro Dry matter digestibility
IVGPT	in vitro Gas production technique
LTE	Long-term experiment
MH	Meadow hay
N	Nitrogen
NDF	Neutral-detergent fiber
NPs	Nanoparticles
NS	Not significant
RF	Ruminal fluid
SCFA	Short-chain fatty acids
SEM	Standard error of the mean
SD	Standard deviation
STE	Short-term experiment
Zn	Zinc

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-024-04281-8.

Supplementary Material 1.

Acknowledgements

The authors are grateful to Valéria Venglovská, Renáta Geročová, and Peter Jerga for laboratory and technical assistance. The publication was financed by the Polish Minister of Science and Higher Education as part of the Strategy of the Poznan University of Life Sciences for 2024-2026 in the field of improving scientific research and development work in priority research areas.

Authors' contributions

D.P., K.M., S.K. and A.B. performed the formal and statistical analyses. D.B. performed the in vitro analyses. K.Č. designed the study protocol and supervised the research. P.K. prepared the phosphate-based Zn nanoparticles. A.L. performed the histological analyses. P.P. and P.S. performed the microbial analyses. A.C. reviewed the ethical application dossier and reviewed the manuscript. Z.V. interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

Funding

This research was funded by the Slovak Research and Development Agency, grant numbers APVV-21–0301 and APVV-SK-PL-23–0004, and by EU NextGenerationEU of the Recovery and Resilience Plan for Slovakia under project No. 09I03-03-V02-00020. This research was also partially funded by the Faculty of Veterinary Medicine and Animal Science, Poznan University of Life Sciences, Poland, by the Department of Animal Nutrition (no. 506.533.04.00). The funders had no role in designing the study; collecting, analyzing, or interpreting the data, or writing the manuscript.

Availability of data and materials

>The data sets used and/or analyzed are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Animal use and study design were conducted following the guidelines of the Declaration of Helsinki and national legislation in the Slovak Republic (G.R. 377/2012; Law 39/2007) for the care and use of research animals. The experimental protocol was approved by the Ethical Committee of the Institute of Animal Physiology, Centre of Biosciences of the Slovak Academy of Sciences on 07 March 2023 (protocol code 1046/2023).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]Centre of Biosciences of Slovak Academy of Sciences, Institute of Animal Physiology, Šoltésovej 4-6, Košice 040 01, Slovak Republic. [2]University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, Košice 041 81, Slovak Republic. [3]Department of Inorganic Chemistry, Faculty of Science, Palacky University, 17. Listopadu 12, Olomouc 779 00, Czech Republic. [4]Department of Preclinical Sciences and Infectious Diseases, Poznan University of Life Sciences, Wolynska 33, Poznan 60-637, Poland. [5]Department of Genetics and Animal Breeding, Poznań University of Life Sciences, Wołynska 33, Poznan 60-637, Poland. [6]Department of Animal Nutrition, Poznan University of Life Sciences, Wolynska 33, Poznan 60-637, Poland.

Received: 6 July 2024 Accepted: 11 September 2024
Published online: 21 September 2024

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