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SHORT COMMUNICATION

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The effect of natural and synthetic zeolites on polysaccharidase activity in the rumen of Jersey heifers

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ABSTRACT. The aim of the present study was to investigate and compare the effects of different types of zeolites and their contribution in the diet on polysaccharidases activity in the rumen of heifers. Natural and synthetic zeolites and their various amounts (2 and 4% dry matter) were used. The study was performed on 5 rumen-fistulated Jersey heifers in a 5 x 5 Latin square design with 5 dietary treatments (control, 2% natural zeolites – ZN2, 2% synthetic zeolites – ZS2, 4% natural zeolites – ZN4, 4% synthetic zeolites – ZS4) and 5 experimental periods. The samples of ruminal digesta were taken from animals before feeding and 3 h after feeding. The significant interactions of diet and sampling time were shown for cellulolytic (P = 0.005), xylanolytic (P = 0.050) and amylolytic (P = 0.044) activities. The addition of 4% ZN to the diet significantly increased pectinolytic activity in the rumen in comparison to control group (P = 0.035). Interestingly, an upward trend of amylolytic activity has been shown (P = 0.082) in ZN2 group. The incorporation of 2% ZS to the diet significantly increased cellulolytic activity compared to control (P = 0.020) and ZN2 diets (P = 0.002). It can be concluded that zeolites can act in the origin- and dose-dependent manner. An increased activity of carbohydrate-digesting enzymes can be related to buffering properties of zeolites, which create favourable conditions for the growth and development of microorganisms. Differences between zeolites action on studied parameters may be derived from their chemical composition and particle size.

Introduction

In recent years, more and more attention has been paid to additives of natural origin, which, on the one hand, exert positive impact of animal health and, on the other hand, are safe for the environment (Majewska et al., 2022). Nutritionists and scientists are still looking for an alternative feeds in ruminant nutrition that like traditional one will be effectively utilized by the animal (Miltko et al., 2024a;b).

Zeolites, sometimes also called ‘magic stones’, are certainly an example of such additives. They are hydrated aluminosilicates with a crystalline structure, in which tetrahedron is a basic unit (Laurino and Palmieri, 2015). In the spatial network of zeolites, a system of channels and chambers can be distinguished, which determines their physicochemical characteristics. The porous structure, high capacity (‘molecular sieves’) and the ability to exchange ions have made zeolites widely used in many fields, including biomedical research and agriculture (Simona and Camelia, 2019). Zeolites possess a negatively charged surface and thus can react with positively charged molecules and neutralize them. The process of adsorption of various molecules (including gases) can occur on

both the inner and outer surfaces of the zeolite and depends mainly on the presence of the channels and chambers of specific size (high selectivity) (Smical, 2011). Depending on the origin, natural and synthetic zeolites can be identified. Natural zeolites are minerals formed from volcanic ash under hydrothermal conditions and are characterized by high stability (pH, temperature) and resistance to acids. The synthetic ones can be artificially produced in the autoclaves by applying minerals to high pressure (Laurino and Palmieri, 2015).

Studies on ruminants have shown that zeolites have the ability to bind toxic substances in feed (pesticide residues, mycotoxins) (Ništiar et al., 2000) or compounds formed as a result of the decomposition of feed with high proportion of carbohydrates (McCollum and Galyean, 1983) and protein (Hemken et al., 1983) (prevention of metabolic diseases). An additional advantage of using these additives is their low cost, which makes them attractive to breeders and they can be an alternative to much more expensive plant extracts. Furthermore, zeolites are also considered to be stable in the gastrointestinal tract (Smical, 2011) and environmentally friendly because of decreasing losses of fecal N (Ghoneem et al., 2022).

To our knowledge the effect of zeolites on the population of the gastrointestinal tract of ruminants in the literature is still unknown. The presence of microorganisms is particularly important because digestion of nutrients in rumen occurs with the contribution of enzymes of microbial origin without the involvement of host enzymes. *In vitro* studies have shown that natural zeolite (clinoptilolite) can be colonized by certain groups of bacteria, constitute their 'microhabitat' and thereby influence biological activity of these bacteria (Weiß et al., 2013). Taking into account the specificity of nutrients digestion in ruminants, the characterization of the effect of zeolites on their abundance and activity seems to be important.

Therefore, the hypothesis of the study assumes that zeolites may affect carbohydrate digestion by modifying microorganisms population in the rumen. The aim of the present study was to investigate and compare the effects of different types of zeolites and their contribution in the diet on polysaccharidases activity in the rumen of Jersey heifers. Two types of zeolites (natural *vs* synthetic) and their various amounts (2% and 4% dry matter (DM)) were used in cow nutrition as a model for ruminants.

Material and methods

All procedures in the present study were approved by the Local Animal Care Ethics Committee for Animal Experiments in Warsaw (Poland); permission no. WAW2/157/2021.

Animals and feeding

The experiment was carried out on 5 rumen-fistulated Jersey heifers with an average body weight of 350 kg in a 5 x 5 Latin square design, in which there were 5 dietary treatments and 5 experimental periods (n = 5). Each period lasted 36 days and comprised 14 days of gradual transition to the diet, 21 days of adaptation to the experimental diet and 1 day of sampling. The animals were housed in the enclosure in the individual stalls (width 155 cm, length 262 cm), allowing for constant visual and olfactory contact between individuals. These stands were litter-free and were equipped with rubber mats. The heifers were able to get up and lie down freely. Keeping cows in the individual stalls was essential to ensure control of feed intake as well as rumen fistula status. Cows had constant access to a trough, salt licks and an automatic waterer. The animals were fed twice a day at 7:00 and 15:00 at the household level. The control animals (CON) were fed (kg/day): meadow hay 6, barley meal 0.8, soybean meal 0.2 and the DOLFOS Dolmix B mineral and vitamin mixture 0.04. Experimental animals also received the addition of natural zeolites (82–86% clinoptilolite, ZeoFEED, ZEOCEM, Slovakia, ZN group) or synthetic zeolites (99% zeolite, ZP-4A, SILKEM, Slovakia, ZS group) in the amount of 2 or 4% per kg DM dose (120 or 240 g/day, respectively) and were divided into four groups: 2% natural zeolites – ZN2, 2% synthetic zeolites – ZS2, 4% natural zeolites – ZN4, 4% synthetic zeolites – ZS4. The crude protein content of tested diets was similar and amounted approximately 10%. Interestingly, clinoptilolite has been classified by the European Union as a dietary supplement for animals (Simona and Camelia, 2019), while zeolite ZP-4A has been approved as a food additive and classified as 'additives other than colours and sweeteners' (E554, according to the manufacturer's information). Daily feed intake was strictly controlled and any appearing orts (if present) were weighed and stored for further analysis.

Rumen digesta sampling

The solid and liquid fractions of ruminal digesta were collected by hand from the dorsal and ventral

sacs of the rumen to obtain representative material. Then, ruminal digesta were precisely mixed, collected to the plastic containers (approximately 100 g) and stored at −24 °C for further enzymatic analyses. The ruminal digesta samples were collected before feeding and 3 h after feeding to observe all the changes that occur over the time.

Chemical analysis of animal diets

Feed samples and orts (if present) were collected during whole experiment for further analyses. The chemical composition of cow diets were analysed according to AOAC International (2011), including DM (934.01), total nitrogen (978.04), crude fat (930.09), neutral detergent fibre (NDF, 2002.04), acid detergent fibre (ADF, 973.18), acid detergent lignin (ADL, 973.18) and crude ash (930.05). The content of the non-fibrous carbohydrate (NFC) was calculated according to NRC (2001) using the following equation:

NFC = 100 – (neutral detergent fibre + crude protein + crude fat + crude ash).

Enzymatic analyses

The method of determination the enzymatic activity in ruminal digesta was based on the assessment of reducing sugars released from each substrate during incubation with enzyme fraction. The extraction of enzymes from approximately 2 g of ruminal digesta (wet weight) was performed with the presence of 20 ml of 1% phosphate buffer (pH = 6), 2.5 ml of carbon tetrachloride and 1 ml of lysozyme solution (50 mg/ml) according to the method of Miltko et al. (2016). Such suspension was incubated at 37 °C for 3 h to obtain liquid fraction, containing enzymes of microbial origin, from each sample. After extraction was done, the liquid fraction was centrifuged at 11 000 x g for 30 min at 4 °C to clear it from digesta particles residues (if present). The next step was incubation at 37 °C for 1 h with the presence of 200 µl of phosphate buffer (pH = 6), 500 µl of purified substrates and 100 µl of clear enzyme fraction. In the present study the low-viscosity carboxymethylcellulose (C5678, Sigma-Aldrich Co., St. Louis, MO, USA), beechwood xylan (X4252, Sigma-Aldrich Co., St. Louis, MO, USA), pectin from citrus (P9125, Sigma-Aldrich Co., St. Louis, MO, USA), inulin (Orafti® HPX, BENEO GmbH, Mannheim, Germany) and potato starch (S2004, Sigma-Aldrich Co., St. Louis, MO, USA) served as substrates for determination cellulolytic, xylanolytic, pectinolytic, inulinolytic and amylolytic activity, respectively. At the end, 1.25 ml of dinitrosalicylic acid reagent (D0550, Sigma-Aldrich Co., St. Louis, MO, USA) was added to the mixture and heated at 100 °C for 5 min to stop enzymatic reaction. The measurement of reducing sugars were quantified spectrophotometrically at 560 nm and their absorbance was compared with the absorbance of glucose, xylose, galacturonic acid and fructose, respectively (Sigma-Aldrich Co., St. Louis, MO, USA). The hydrolytic activity of the enzyme fraction was expressed as equivalent of appropriate sugar per g of DM of ruminal digesta per min. To determine the DM of digesta, ruminal samples were dried at 60 °C for 24 h and then at 105 °C for the next 24 h.

Statistical analysis

The results are presented as means with standard error of mean (SEM). The Shapiro-Wilk test was used to check the normality of data. The homogeneity of variances was also performed using the Levene's test. Data obtained were subjected to repeated measures analysis of variance (ANOVA) with diet (CON, ZN2, ZS2, ZN4, ZS4), sampling time (0, 3 h) and their interactions as main effects, followed by Tukey's post-hoc test. To check the correctness of the scheduled experiment, the effect of animal (1–5) and period (I–V) on results obtained were also verified. The significance between means were assumed at $P \leq 0.05$ and trends at $0.05 < P < 0.10$ (StatSoft®, Cracow, Poland).

Results and discussion

The composition of cow diets is presented in Table 1. Dry matter intake ranged from 6 184 (ZS4 group) to 6 487 g/day (ZN4 group), depending on dietary treatment. Other ingredients intake were similar in all dietary treatments excluding ZS4 group, in which the orts constituted 5.23% of the total dose. For comparison, orts in other experimental groups were as followed (%): 1, 0.78 and 0.51 for ZN2, ZN4 and ZS2 diets, respectively. Deterioration of feed intake by cows from ZS4 group may be due to too high contribution of synthetic zeolites in a diet.

Table 2 presents the polysaccharidase activity in the ruminal digesta of Jersey heifers.

The statistical analysis showed that both animal (1–5, P_A) and experimental period (I–V, P_p) did not affect obtained results for cellulolytic ($P_A = 0.727$; $P_p = 0.395$), xylanolytic ($P_A = 0.692$; $P_P = 0.111$), pectinolytic ($P_A = 0.200$; $P_P = 0.760$), inulinolytic ($P_A = 0.723$; $P_P = 0.837$) and amylolytic ($P_A = 0.647$; $P_P = 0.598$) activities. The significant interaction of

Table 1. Composition of cow diets

Item	Control	Natural zeolites (ZN)		Synthetic zeolites (ZS)	
		ZN2	ZN4	ZS2	ZS4
Components, g/kg DM					
meadow hay	5397	5397	5397	5397	5397
barley meal	698	698	698	698	698
soybean meal	178	178	178	178	178
Dolfos[1]	39.0	39.0	39.0	39.0	39.0
ZeoFEED	-	113	226	-	-
ZP-4A	-	-	-	107	214
Chemical composition, g/kg DM					
DM	897	897	898	896	896
crude protein[2]	94.3	92.7	91.2	92.7	91.2
crude fat	23.0	22.6	22.2	22.6	22.2
crude ash	37.6	37.0	36.3	37.0	36.3
crude fibre	271	266	262	266	262
NDF	599	588	579	588	579
ADF	352	346	340	346	340
ADL	59.9	58.9	57.9	58.9	57.9
NFC	241	237	233	237	233
Nutrient intake, g/day					
DM	6312	6361	6487	6385	6184
crude protein	664	657	659	661	629
crude fat	162	160	161	161	154
crude ash	265	262	263	264	251
crude fibre	1905	1886	1890	1895	1805
NDF	4214	4172	4181	4193	3994
ADF	2478	2453	2459	2465	2348
ADL	422	418	419	420	400
NFC	1697	1680	1684	1684	1608

DM – dry matter, NDF – neutral detergent fibre, ADF – acid detergent fibre, ADL – acid detergent lignin, NFC – non-fibrous carbohydrate; ZeoFEED – natural zeolite, ZP-4A – synthetic zeolite, Zn2 – 2% of natural zeolites, ZN4 – 4% of natural zeolites, ZS2 – 2% of synthetic zeolites, ZS4 – 4% of synthetic zeolites; [1] Dolfos DOLMIX B consisted of: calcium carbonate, sodium chloride, calcium-magnesium carbonate, mono-calcium phosphate, magnesium oxide, glycerol; UI: vit. A 700 000, vit. D_3 140 000; mg: vit. E 1 650, niacin 500, DL-α-tocopherol 1 500, trace elements, mg: copper 30, manganese 60, zinc 1000, selenium 30; g: sulphur 5.5, calcium 253, sodium 80, magnesium 30, phosphorus 10; [2] expressed as N x 6.25

both experimental factors (diet and sampling time) was shown for cellulolytic (P = 0.005), xylanolytic (P = 0.050) and amylolytic (P = 0.044) activities. In terms of sampling time, the inclusion of 2% ZS to cow diets increased cellulolytic (P = 0.015) and amylolytic (P = 0.048) activities 3 h after feeding in comparison to samples collected before feeding. Similarly, the addition of ZN at low and high doses to cow diets increased inulinolytic (P = 0.029) and amylolytic (P = 0.002) activities in the rumen after feeding, respectively.

Generally it was shown that dietary treatment significantly increased enzymes activity 3 h after feeding. The exception was only reduced amylolytic activity before feeding in heifers receiving 4% ZN in the diets in comparison to control animals (P = 0.069).

The addition of 4% ZN to the diet significantly increased pectinolytic activity in the rumen in comparison to control group (P = 0.035). Interestingly, when smaller dose of ZN was used, an upward trend of amylolytic activity has been shown (P = 0.082). On the other hand, the incorporation of 2% ZS to animal diets significantly increased cellulolytic activity compared to control (P = 0.020) and ZN2 diets (P = 0.002). Comparing both types of zeolites used in the present study, the decreased inulinolytic activity in the rumen of ZS2 heifers in comparison to ZN2 (P = 0.009) and control groups (P = 0.083) was observed.

Natural and synthetic zeolites significantly affected microbial enzymes digesting both structural (cellulose, pectin) and soluble (amylose, inulin) carbohydrates. In the present study the action of zeolites on polysaccharidases seems to be origin- and dose-dependent. Higher activity of these enzymes in zeolite-supplemented cows was probably related to their buffering capacity, which can be associated with the presence of aluminium silicate in their structure (Khachlouf et al., 2018). Interestingly, bacterial cell walls are negatively charged under physiological conditions, which allows for interacting with cations present on the surface of zeolites (Guo et al., 2011). Therefore, zeolites may stabilize rumen pH, provide necessary cations for the activity of specific bacteria and, in consequence, enhanced nutrients digestion (Mahdavirad et al., 2021). Due to their ion chelating properties (connected with the presence of iron and zinc), these compounds can significantly influence the basic biochemical transformation occurring in bacterial cells (Trckova et al., 2004). Zeolites can also improve fibre digestion by gradual releasing ammonia due to ion exchange across the sodium and potassium (Ural and Ural, 2017). The role of protozoa in the digestion of nutrients in the rumen should not be omitted, because they are also able to digest structural and soluble carbohydrates. However, the effect of zeolites on the protozoa population is still unknown.

The results of the current study are in agreement with Galindo et al. (1990), who noted that *in vitro* study on zeolites increased cellulolytic activity of microorganisms without any effect on haemicellulolitic activity. Furthermore, the study of Kardaya et al. (2023) showed that both Indonesia's natural zeolites and urea-impregnated zeolites added to sheep diet improved the digestibility

Table 2. Polysaccharidase activity in the ruminal digesta of Jersey heifers, µM released sugar/g DM/min

Enzyme activity	Diet (D)	Sampling time (T)		$Mean_D$	SEM_D	*P*-value		
		0h	3h			D	T	D x T interaction
Cellulolytic[1]	Control	14.1	11.3^{A}	12.7	1.055	0.020	0.015	0.005
	ZN2	12.8	9.44^{A}	11.1	1.027			
	ZS2	10.3_X	$16.7^{B}{}_Y$	13.5	1.451			
	ZN4	16.3	13.3^{AB}	14.8	1.019			
	ZS4	11.5	13.4^{AB}	12.5	0.964			
	$Mean_T$	13.0	12.8					
	SEM_T	0.775	0.677					
Xylanolytic[2]	Control	38.6	36.0	37.3	0.974	0.645	0.505	0.050
	ZN2	31.4	36.3	33.9	1.620			
	ZS2	34.7	35.9	35.3	1.041			
	ZN4	36.5	35.3	35.9	1.723			
	ZS4	38.5	32.6	35.5	1.687			
	$Mean_T$	35.9	35.2					
	SEM_T	1.005	0.807					
Pectinolytic[3]	Control	2.78	2.76^{A}	2.77	0.125	0.035	0.098	0.529
	ZN2	2.85	3.21^{AB}	3.03	0.180			
	ZS2	2.73	2.86^{AB}*	2.79	0.126			
	ZN4	3.03	3.55^{B}*	3.29	0.185			
	ZS4	2.97	2.98^{AB}	2.98	0.190			
	$Mean_T$	2.87	3.07					
	SEM_T	0.117	0.092					
Inulinolytic[4]	Control	2.81	3.51^{AB}*	3.16	0.234	0.024	0.029	0.500
	ZN2	2.79_X	$3.97^{B}{}_Y$	3.38	0.252			
	ZS2	2.22	2.31^{A}*	2.27	0.159			
	ZN4	2.81	3.19^{AB}	3.00	0.318			
	ZS4	2.98	3.15^{AB}	3.07	0.220			
	$Mean_T$	2.72	3.22					
	SEM_T	0.150	0.168					
Amylolytic[5]	Control	4.40*	3.46*	3.93	0.302	0.282	0.002	0.044
	ZN2	3.64	5.49*	4.57	0.507			
	ZS2	3.32_X	5.26_Y	4.29	0.508			
	ZN4	2.84_X*	4.83_Y	3.84	0.391			
	ZS4	4.08	4.94	4.51	0.262			
	$Mean_T$	3.66	4.80					
	SEM_T	0.195	0.258					

D – effect of diet, T – effect of sampling time, D x T – diet and sampling time interaction effect, Zn2 – 2% of natural zeolites, ZN4 – 4% of natural zeolites, ZS2 – 2% of synthetic zeolites, ZS4 – 4% of synthetic zeolites; SEM – standard error of the mean; [1] expressed as µM glucose released/g DM/min; [2] expressed as µM xylose released/g DM/min; [3] expressed as µM glucuronic acid released/g DM/min; [4] expressed as µM fructose released/g DM/min; [5] expressed µM glucose released/g DM/min; [A,B] – means with different superscripts in a column are significantly different at $P \leq 0.05$ between diet (control, ZN2, ZS2, ZN4, ZS4); * – means with a star in a column are used to mark an appearing trends at $0.05 < P < 0.10$; [X,Y] – means with different letters in a row are significantly different at $P \leq 0.05$ between sampling time (0, 3 h)

of DM, organic matter (OM), NDF, ADF and haemicellulose as well as body weight gain and feed efficiency. Increased fibre digestion was also obtained by Ghoneem et al. (2022), when 2% natural zeolites were added to lamb diets. On the other hand, McCollum and Galyean (1983) showed that 1.5% clinoptilolite supplemented to beef steers' high-grain diet increased starch digestion and modified rumen fermentation by increasing concentration of propionate. Similar dependencies were noted in the present study, when lower dose of ZN was added to cow diets. According to Valpotić et al. (2017), both natural and synthetic zeolites may affect energy metabolism due to changes in rumen fermentation and molar proportion of short-chain fatty acids. Our unpublished data showed that higher doses

of both types of zeolites increased concentrations of acetate and propionic acids, which supports increased polysaccharidase activity observed in the present study.

The various effect of natural and synthetic zeolites on enzyme activity can be derived from different chemical composition, which can determine their physicochemical properties. Clinoptilolite, as an example of natural zeolite used in this study contained 62% SiO_2, 14% Al_2O_3, 2.3% Fe_2O_3 and 5.5% CaO, while ZP-4A (synthetic zeolite) contained 17–19% Na_2O, 28–30% Al_2O_3 and 31–34% SiO_2 (according to the manufacturer's information). The particle size of the zeolites used also seemed to be important (200 μm for clinoptilolite *vs* 3–5 μm for ZP-4A), because it could strongly affect their selectivity to adsorbate molecules (Bacakova et al., 2018). In the study of Hrenović et al. (2008), the number of immobilised cells of *Acinetobacter junii* (phosphate-accumulating bacteria) were higher in zeolites with smaller particle size, which in consequence increased their activity. Klaeuli et al. (2020) showed that digestibility of organic matter was higher, when clinoptilolite of 30 μm particle size was supplemented to backgrounding cattle than that of 400 μm. Similarly, the study of El-Nile et al. (2021) on goats showed that transformation of natural zeolites to the nano-size has enhanced their physicochemical properties, reduced concentration of NH_3-N and production of CH_4 without any detrimental effects on nutrients digestibility.

Conclusions

The results of the present study showed that zeolites can act in the origin- and dose-dependent manner. Low dose of natural zeolites increased amylolytic activity, while its higher dose significantly increased pectinolytic activity in the rumen. On the other hand, in cows receiving low dose of synthetic zeolites, higher cellulolytic activity was observed. An increased activity of carbohydrate-digesting enzymes can be related to buffering properties of zeolites, which create favourable conditions for the growth and development of microorganisms. All occurring differences between zeolites action on studied parameters may be derived from their chemical composition and particle size, which can significantly determine physicochemical properties of these compounds.

So, it is recommended to use up to 2% zeolites of both types in cow diets to improve microbial enzymes activity digesting both structural and soluble carbohydrates. The higher dose of synthetic zeolites (4%) incorporated to the diet, seemed to be less effective and profitable due to reduced feed intake. However, further studies on a larger number of animals are necessary to fully understand the mechanisms of actions of zeolites on microorganisms population and digestive processes in ruminants.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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