Comparative in vitro study of caecal microbial activity in brown hares and domestic rabbits which were offered the same diet

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Abstract

The products of the microbial activity in the large intestine are an important source of energy for herbivores. A previous study showed differences in caecal methanogenesis and short-chain fatty acid (SCFA) profiles between brown hares and domestic rabbits. The present study was performed on animals which were offered the same diet to eliminate the impact of diet on the comparative analyses of microbial metabolites. Caecal samples of hares and rabbits were incubated in triplicate, i.e. without any supplementary substrate (control) or with the addition of wheat bran or oat bran. Calculated as percentage of body weight, the stomachs of the rabbits were heavier than those of the hares, but caeca of the hares weighed more than those of the rabbits. The total SCFA concentration in caecal samples was higher in rabbits than in hares, and it increased in both species when the supplementary fermentation substrates were added. In hares, the molar proportion of propionate was higher and that of butyrate was lower compared to rabbits. The addition of substrate decreased acetate and propionate but increased the molar proportion of butyrate. Microbial fermentation resulted in greater gas release in rabbit caecal samples compared to those of hares. Methanogenesis tended to be lower in hares than in rabbits, but high individual variability was observed, especially in hares. Our study stated lower microbial activity in the caeca of brown hares compared to domestic rabbits. The presented results might lead to assumption that differences between fermentation patterns were not caused by diet but resulted from the peculiarities of both species.

Keywords European hare · Domestic rabbit · Caecum · In vitro fermentation · Short-chain fatty acids · Methane

Introduction

Microbial processes in the digestive tract are important sources of energy and nutrients, especially for herbivores. The intestinal microbiota breaks down indigestible dietary components, particularly fibre, and produces fermentation metabolites such

as short-chain fatty acids (SCFAs), which mainly include acetate, propionate and butyrate. SCFAs are one of the essential energy sources for herbivorous mammals, but they also have physiological, such as immunomodulatory, regulatory and metabolic, functions as well as actions on gut motility, morphology and function (Bergman 1990; Hurst et al. 2014; Kasubuchi et al. 2015; Corrêa et al. 2016). Microbial fermentation of carbohydrates is accompanied by the production of gases, including carbon dioxide, hydrogen and methane, and the methanogenic Archaea is one of the microbial groups competing to uptake metabolic hydrogen. The level of methanogenesis in the gastrointestinal tract of an animal depends on several connected factors that are specific to the intestinal ecosystem of each species, but it also depends on such factors as individual features, current physiological conditions and diet (Christl et al. 1992; Tiemann et al. 2008; Franz et al. 2010; Hook et al. 2010; Belenguer et al. 2011; Abecia et al. 2013).

Due to the importance of microbial activity, fermentation chambers often constitute a substantial portion of animal digestive tracts, and some small herbivorous mammals have an

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enlarged caecum as the main fermentation chamber, which is why they are classified as hindgut fermenters. Because these animals require much more energy and protein per unit body mass than larger herbivores, they employ characteristic digestive system strategies to overcome the limitations of their small body mass to obtain enough energy and nutrients to survive (Sakaguchi 2003).

Leporids are small mammalian herbivores that select food to obtain the necessary amount of nutrients and energy for their bodies, and despite similarities in size and appearance, hares (*Lepus* spp.) and rabbits (*Oryctolagus* spp.) differ in their feeding strategy and digestive physiology. The ability to digest nitrogenous compounds is greater in rabbits than in hares (Kuijper et al. 2004), and similarly, the digestibility of hemicelluloses is higher in rabbits than in hares, although both species are poor digesters of fibre (Stott 2008). In cases where only low-quality forage is available, hares maximise food processing by increasing their intake rate and decreasing the digesta retention time, while rabbits maximise digestion by increasing the mean digesta retention time (Kuijper et al. 2004). The gut passage rate has been found to be significantly faster in hares than in rabbits (Stott 2008). Another adaptive approach in leporids to cope with low-quality forage is caecotrophy, the ingestion of special soft faeces originated from caecal content. Due to a mechanism that separates poorly digestible fibres from the digestible food fraction in the colon, the fibrous fraction is excreted as hard faeces, whereas the remaining digestible fraction provides material to form soft faeces, which are consumed and reingested after excretion. The reingestion of soft faeces provides additional protein, minerals and vitamins to the animal (Hirakawa 2001), but this mechanism is not identical in both rabbits and hares. Rabbits are better able to separate the fibrous fraction and to produce larger amounts of soft faeces as a cluster surrounded by a mucilaginous membrane than hares, which produce an amorphous type of soft faeces that lacks a membrane (Hirakawa and Okada 1995; Hirakawa 2001; Kuijper et al. 2004).

The microbial activity in the caecum of European rabbit (*Oryctolagus cuniculus*), especially its domesticated varieties, has been largely described in the previous literature in terms of the SCFA profile and methane levels, which are the main products of the caecal microbiota (Bellier et al. 1995; Piattoni et al. 1996; Marounek et al. 1999; Garcia et al. 2002; Bennegadi et al. 2003; Mišta et al. 2011), but information on the caecal microbiota activity of the hare remains insufficient. Our previous research, performed in vitro using caecal inocula obtained from brown hares (*Lepus europaeus*) from a natural environment and caged domestic rabbits, showed some differences in the SCFA profile of these animals, suggesting different activities of the bacteria inhabiting their caeca (Marounek et al. 2013; Mišta et al. 2015). These differences were mainly focused on total SCFA concentration, which was higher in rabbits compared to hares, as well as the propionate/butyrate ratio, which was higher

in hares than in rabbits. In our previous experiments, the most surprising result was that methane production was very low in hare caecal samples compared to those of rabbits, and we established that caecal methanogenesis in the hare remained very low despite the addition of substrate into inoculums (Mišta et al. 2015). However, the impact of diet, which differed between rabbits and hares, on the variations in the products of microbial activity between species was uncertain.

Our present study of brown hares and domestic rabbits which were offered the same diet should resolve the above question as well as dispel doubts regarding the methodological approach and the dissimilarities in the production of microbial metabolites in the caecum in these two species.

Materials and methods

Experimental animals

Six female and four male brown hares, aged 7 months and weighing approximately 2.4 kg, were obtained from the Caged Hare Breeding Unit (Paszów, Poland), and six female New Zealand White rabbits and four males aged 4 months and weighing approximately 2.9 kg were also used (Center for Experimental Medicine, Medical University of Silesia, Katowice, Poland). Differences in the ages of the rabbits and hares in the experiment were due to the distinct ages of sexual maturity of these species. During the experiment, all animals were housed in individual cages with free access to water, meadow hay and a homogenised pelleted diet (Table 1), which

Table 1 The composition of the basal feed (pelleted) mixture (g/kg)

Ingredients	
Dried grass	130
Dried alfalfa	150
Wheat bran	170
Wheat grain	100
Barley grain	100
Corn	150
Extracted soybean meal	100
Rape meal	50
Rapeseed oil	10
Vitamin-mineral premix ^a	40

^aPremix provided per kg of diet: vitamin A, 15000 IU; vitamin D₃, 1504.0 IU; vitamin B₁, 8.7 mg; vitamin B₂, 13.1 mg; vitamin B₆, 7.1 mg; vitamin B₁₂, 0.01 mg; vitamin E, 57.2 mg; vitamin C, 73 mg; vitamin K, 39.9 mg; folic acid, 1.7 mg; niacin, 63.10 mg; pantothenic acid, 30.1 mg; choline, 884 mg; inositol, 1334 mg; biotin, 0.272 mg; Mn, 80 mg; Cu, 15 mg; Fe, 197 mg; Zn, 62 mg; Co, 0.523 mg; iodine, 0.638 mg; Se, 0.157 mg; F, 0.032 mg

was formulated according to the nutritional requirements of rabbits (de Blas and Wiseman 1998). Hares were kept in individual wooden outdoor pens under natural daylight and temperature, and rabbits were housed in standard stainless steel cages, one animal per cage, at 20 ± 2 °C.

Before and after the 6-week experimental diet period, all animals were weighed, and parasitological examinations of faeces were performed. Faecal samples were examined for presence of gastrointestinal parasites (protozoa and helminths) by flotation using the diagnostic kit Fecalyzer/Fecasol (Vetoquinol Biowet, Gorzów Wielkopolski, Poland) according to the manufacturer's instructions. The McMaster counting technique was used to determine the number of eggs/oocysts present per gram of faeces (e.p.g.).

After 6 weeks, the animals were slaughtered (using xylazine and sodium pentobarbital injections), and the stomachs and caeca were then resected to chemically analyse the gastric digesta and perform *in vitro* fermentation of the caecal contents. The organs were emptied to collect the digesta for further analyses. The euthanasia and sampling took place about 6 p.m. All the empty stomachs and caeca were weighed, and their percentages of the final body weight were calculated.

In vitro fermentation

Immediately after resection, the caecal contents were squeezed out and mixed, and their pH was measured. Three 20-g sub-samples were taken from each caecal sample and transferred into 125-ml serum bottles, and each sub-sample was diluted in 80 ml of buffer solution (pre-warmed to 39 °C, pH 7.2), which was prepared according to Adjiri et al. (1992). All sub-samples were prepared for incubation either without any supplementary substrate (C, control samples) or by adding 1 g of one of the substrates (WB, samples with wheat bran; OB, samples with oat bran). Before incubation, each serum bottle was thoroughly flushed with CO₂ to obtain anaerobic conditions and then hermetically sealed with rubber stoppers and aluminium caps using a manual crimper. All manipulations of the caecal digesta were performed as quickly as possible (only a few minutes for each caecum), and the incubation was performed in a shaking water bath at 39 °C for 12 h. The wheat bran, rich in hemicelluloses, the important components of rabbits' feeds, was used as one of the supplementary fermentation substrates. The brown hare is known to select plant parts that are rich in fat (Homolka 1987; Smith et al. 2005; Popescu et al. 2011; Schai-Braun et al. 2015), so oat bran, which is approximately twice as rich in fat as wheat bran, was chosen as the second fermentation substrate.

Methane and total gas release

At the end of incubation, the headspace pressure inside each bottle was measured using a manual pressure manometer

connected to a needle that was used to punch the rubber stopper, and the fermentation gas was subsequently sampled with a gas-tight syringe for the analyses. The total gas release was calculated using the Clapeyron equation (ideal gas law) based on the gas pressure, the headspace volume and the temperature inside the serum bottle. The sampled fermentation gas was analysed for methane using a 7890A gas chromatograph (Agilent Technologies, Santa Clara, USA) equipped with a flame ionisation detector; a thermal conductivity detector; two Supelco columns, Porapak Q and HayeSep Q (Supelco, Bellefonte, USA); and a 5A molecular sieve, and helium was used as the carrier gas (flow rate 25 ml/min). Based on the gas pressure and the percentage of methane in the total gas volume, the molar concentration of methane was calculated using the Clapeyron equation.

Short-chain fatty acid analysis

After incubation, the pH of the incubation fluid was measured, and the fermentation was then stopped by adding 0.05 ml of formic acid per 1 ml of fluid. The samples were centrifuged (2000g for 15 min) and analysed for SCFA with a 7890A gas chromatograph (Agilent Technologies, Santa Clara, USA) with a flame ionisation detector and an Agilent DB-WAX UI J & W column with helium as the carrier gas (flow rate 25 ml/min). This analysis was performed to determine the caecal concentrations of acetate, propionate, isobutyrate, butyrate, isovalerate, valerate and caproate. The standard curves were prepared using Volatile Free Acid Mix (Supelco), and the total SCFA concentration was calculated as the sum of the SCFA concentrations in the digesta.

Chemical analyses of the gastric contents, food and fermentation substrates

Because hares produce amorphous soft faeces that is barely distinguishable from other materials in the stomach ingesta, all animal gastric samples contained caecotrophs and were mixed before analysis of the chemical composition. The collected samples of hare and rabbit gastric content as well as samples of pelleted food, meadow hay, wheat bran and oat bran were analysed using standard methods for dry matter (DM; AOAC 2005 method 934.01), crude protein (CP; Kjeldahl method, AOAC 2005 method 984.13), ether extract (EE; AOAC 2005 method 920.39), crude fibre (CF; AOAC 2005 method 978.10), ash (AOAC 2005 method 942.05), neutral detergent fibre (NDF; method of Holst 1973) and acid detergent fibre (ADF; AOAC 2005 method 973.18). The gross energy in the feed samples was measured using an adiabatic oxygen bomb calorimeter KL-10 (Precyzja, Bydgoszcz, Poland), and the non-structural carbohydrate (NSC) content was calculated according to the National Research Council (2001) as follows:

100 – (ash + CP + EE + NDF), where ash, CP, EE and NDF contents were expressed as percentages of the DM.

Statistical analyses

The gastric content data were subjected to a one-way analysis of variance (ANOVA) using the STATISTICA version 12.5 software package for MS Windows to compare the rabbit and hare results, and a two-way ANOVA was used to compare the in vitro fermentation parameters of the rabbit and hare caecal inoculums. The effects of species, the addition of a substrate to the incubated samples and their interaction (species $\times$ substrate) on the fermentation parameters were evaluated. The stomach and caecal weight data were also subjected to a two-way ANOVA to analyse the effects of species, sex and their interaction (species $\times$ sex). Differences between means were considered statistically significant if $P < 0.05$.

Data availability The datasets during and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

Animal and organ weights

Table 2 shows that the initial body weights of the hares differed from those of the rabbits even though the experimental animals of both species were near sexual maturity. Rabbits were heavier than hares during the experiment, and that difference increased due to higher body weight gain by the rabbits (approximately 22% of the initial body weight) compared to the hares (9%) (Table 2). In addition, the females of both species gained more weight than males.

As expected, the weight of the stomach as a percentage of the total body weight, for both the empty organs and including the weight of the digesta, was greater in rabbits than hares ($P < 0.001$) (Table 3), and the stomachs of females were heavier than the stomachs of males ($P < 0.05$). Surprisingly, as shown in Table 3, the empty caeca of hares (% BW) weighed significantly more than the empty caeca of rabbits ($P < 0.05$), and the

hare caecal digesta (% BW) was, on average, one and a half fold heavier than the rabbit caecal digesta ($P < 0.001$). There was no statistically significant effect of sex on caecal weights.

Parasitological examination of the faecal samples of hares and rabbits revealed the presence of protozoa of the genus *Eimeria*, but the number of oocysts per gram of faeces was below the detection of the McMaster technique (less than 50 o.p.g.).

Chemical analyses

The results of the chemical analyses of the diet and fermentation substrates used in the experiment are summarised in Table 4, and the comparison of the chemical composition of the rabbit and hare stomach digesta is presented in Table 5. The composition of the digesta did not differ significantly between the two species, but the percentage of dry matter in hares tended to be lower while the ash content tended to be higher.

There were differences in the substrates used for in vitro fermentation, mainly in the contents of fibre, ether extract and non-structural carbohydrates. The oat bran was markedly poorer in crude fibre, NDF and ADF but approximately two-fold richer in fat and NSC than wheat bran. The differences in fibre content were primarily due to ADF, which was more than fourfold higher in the wheat bran, whereas crude fibre and NDF were threefold higher in the wheat bran compared to the oat bran.

Caecal fermentation profile

Based on our results, both the species ($P < 0.01$) and the type of substrate ($P < 0.001$) significantly affected the production of SCFA in the caecal digesta (Table 6). The total SCFA concentration approximately doubled when wheat bran was used as the fermentation substrate, and it increased from two and a half fold (in rabbits) to almost threefold (in hares) when oat bran was used. Comparing the production of SCFA between the examined species, the results indicated that the total concentration of bacterial metabolites was more than 20% higher in rabbit caecal digesta than that of hares.

The proportions of the three main SCFAs, the main parameters characterising the fermentation pattern in the animal caecum, differed significantly between rabbits and hares (Fig. 1). When the sum of the concentrations of the three main acids was taken as 100%, acetate presented a similar proportion in both species: 71% in rabbits and 69% in hares. The proportions of propionate and butyrate were 5 and 24%, respectively, in rabbits, and the proportions were 17 and 15% in hares. The molar proportions of both of these SCFAs varied significantly between species ($P < 0.001$), which resulted in differences in the acetate/propionate and propionate/butyrate ratios ($P < 0.001$) (Table 6). The acetate/propionate ratio calculated for rabbits was approximately threefold higher than that in

Table 2 Body weight and body weight gain of brown hares and domestic rabbits

Item	Rabbit		Hare		SEM
	Male	Female	Male	Female	
Initial body weight, kg	2.879	2.823	2.503	2.270	0.084
Final body weight, kg	3.286	3.570	2.653	2.518	0.119
Body weight gain, kg	0.41	0.75	0.15	0.25	0.059

Table 3 Stomach and caecal weights of brown hares and domestic rabbits

Item	Rabbit		Hare		SEM	P value		
	Male	Female	Male	Female		Species	Sex	Species × sex
Stomach tissue weight, %BW	0.90	1.14	0.71	0.79	0.047	< 0.001	0.014	0.184
Stomach digesta weight, %BW	2.32	2.63	0.64	1.13	0.245	< 0.001	0.277	0.801
Caecal tissue weight, %BW	1.49	1.62	1.76	2.03	0.085	0.049	0.214	0.650
Caecal digesta weight, %BW	2.16	2.79	3.67	4.14	0.227	< 0.001	0.112	0.818

%BW percentage of body weight

hares, whereas the propionate/butyrate ratio calculated for rabbits was approximately sevenfold lower than that for hares. The caecal fermentation profile of the two species also differed in the caproate content ($P < 0.001$), which was very low in hares, approximately 0.02 mol% on average, whereas it was 0.3 mol% in rabbits (Table 6).

The addition of a supplementary substrate decreased acetate molar proportion ($P < 0.001$) to a greater extent in rabbits than in hares (Table 6), and in rabbits, oat bran caused a greater reduction in acetate than wheat bran. In hares, both substrates similarly affected the proportion of acetate. Due to these changes, there was also a statistically significant species × substrate interaction ($P < 0.01$), and the substrate also decreased the molar proportion of propionate ($P < 0.05$) and increased that of butyrate ($P < 0.001$), which caused a decrease in the propionate/butyrate ratio ($P < 0.01$) (Table 6). In general, these changes were more pronounced after the addition oat bran compared to wheat bran, but there were some species-specific differences because wheat bran did not affect the molar proportion of propionate in rabbits.

Although SCFA concentrations were higher in rabbits, pH values in incubated caecal samples were, on average, lower in hares ($P < 0.001$), but this did not apply to fresh, undiluted caecal samples, in which the difference between species was minimal (Table 6). In the samples incubated with supplementary substrates, lower pH values were observed, and oat bran had a more pronounced effect ($P < 0.001$).

Total gas release and methanogenesis

Microbial fermentation resulted in greater gas release in rabbit caecal content compared to that of hares ($P < 0.001$) (Table 6). Similar to SCFA production, total gas release increased when supplementary substrates were added to caecal cultures, and the greatest amount of gas production was observed in samples incubated with oat bran ($P < 0.001$). The addition of substrate significantly increased the production of gas in the caecal cultures of both rabbits and hares, but the measured increase in methane production in the cultures of both species was not significant.

Discussion

Digestive organ weights

Body size is known to influence the feeding strategies and digestive ability of herbivorous animals. A larger body size promotes gastrointestinal retention and digestive capacity, whereas a smaller size promotes selective feeding behaviour because of the shorter mean retention time due to a smaller digestive tract (Van Soest 1996). As small herbivores classified as concentrate selectors, rabbits select the young parts of plants, which are relatively poor in fibre and rich in protein. In contrast, hares are classified as intermediate feeders that eat browse material when the availability of higher-quality feed is

Table 4 Chemical analysis of animal feed and fermentation substrates

Item	Pelleted feed	Meadow hay	Wheat bran	Oat bran
Dry matter, %	90.93	90.35	93.38	92.78
Ash, %DM	6.32	8.05	5.09	2.83
Crude protein, %DM	17.30	9.17	17.98	15.78
Crude fibre, %DM	8.19	31.96	12.6	3.72
Neutral detergent fibre, %DM	25.71	66.05	47.2	13.9
Acid detergent fibre, %DM	8.76	36.95	18.44	3.94
Ether extract, %DM	3.04	3.55	4.23	9.42
Non-structural carbohydrates, %DM	47.63	13.18	25.5	58.07
Gross energy, MJ/kg	16.88	17.47	16.22	16.61

%DM percentage of dry matter

Table 5 Chemical composition of gastric content

Item	Rabbit's gastric content	Hare's gastric content	SEM	<i>P</i> value
Dry matter, %	20.6	17.5	0.813	0.053
Ash, %DM	5.62	8.95	0.932	0.073
Crude protein, %DM	14.1	13.5	0.830	0.733
Crude fibre, %DM	24.7	22.6	1.304	0.437
Neutral detergent fibre, %DM	53.1	47.8	2.306	0.257
Acid detergent fibre, %DM	27.6	26.8	1.527	0.791
Ether extract, %DM	3.4	3.2	0.334	0.793
Non-structural carbohydrates, %DM	23.7	26.5	2.242	0.547
Gross energy, MJ/kg DM	18.2	19.0	0.406	0.360

%DM percentage of dry matter

limited (Hulbert et al. 2001). Moreover, some researchers have suggested that the brown hare's diet is an adaptation to maximise energy intake by consuming a diet rich in fat (Schai-Braun et al. 2015).

In herbivorous mammals, the capacity of the digestive tract increases in proportion to body weight, but the corresponding increase in energy requirements is generally three-quarter power of the body weight (Van Soest 1996). This could partly explain differences in the development of digestive organs and their weights as a proportion of the body weight between related but differently sized species. Previous studies of

leporids reported heavier digestive organs in European rabbits compared to brown hares, which was explained as an adaptation by hares to achieve greater weight minimisation to maximise running speeds to escape predators (Stott 2008). As predicted, the weight of the stomach tissue in hares was also lower than that of rabbits in our experiment, which was consistent with the data from the literature (Klein and Bay 1994). Stott (2008) reported that the stomachs of rabbits (together with the digesta) were approximately twice as heavy as those of hares when the organ weights were calculated as percentages of the gross carcass weights. Similarly, the stomach digesta in our experiment weighed less in hares than in rabbits, which, in addition to the smaller organ proportion, could be explained by the faster passage of the digesta in hares compared to in rabbits (Kuijper et al. 2004; Stott 2008). Surprisingly, both the caecal tissue and digesta were significantly heavier in our hares than in our rabbits, which was inconsistent with the data of Stott (2008) and may be explained by that author's use of wild-type rabbits, which had a different body mass (the hare carcasses weighed almost twice as much as the rabbit carcasses) and thus probably different organ weight proportions than those of domestic rabbits. It is worth noting that the mean absolute empty caeca weights were quite similar among the animals in our experiment (49 g in hares and 54 g in rabbits). Thus, it is possible that among related species of caecal fermenters, smaller animals need similarly sized fermentation chambers for microbial processes as larger animals because of their high energy

Table 6 Short-chain fatty acids, pH values and gas production in caecal samples after 12-h in vitro fermentation

Fermentation parameters	Rabbit			Hare			SEM	<i>P</i> value		
	C	WB	OB	C	WB	OB		Species	Substrate	Species × substrate
Total SCFA, mmol/kg	160.3	312.1	396.1	110.2	241.9	321.8	16.66	0.007	<0.001	0.902
SCFA, mol%:										
Acetate	74.34	66.37	63.62	67.58	65.65	65.32	0.977	0.079	<0.001	0.007
Propionate	5.71	5.92	4.28	18.36	16.63	13.27	0.756	<0.001	0.047	0.398
Isobutyrate	0.69	0.44	0.62	2.09	0.62	0.39	0.154	0.251	0.113	0.207
Butyrate	17.29	24.78	27.29	9.78	14.38	18.18	0.915	<0.001	<0.001	0.667
Isovalerate	1.11	0.94	0.96	1.41	1.23	1.46	0.118	0.206	0.872	0.947
Valerate	0.76	1.33	2.67	0.78	1.47	1.34	0.266	0.562	0.335	0.619
Isocaproate	ND	ND	0.06	ND	ND	ND	0.017	0.322	–	–
Caproate	0.18	0.21	0.50	ND	0.01	0.04	0.036	<0.001	0.075	0.207
Acetate/propionate	13.80	11.95	15.39	4.56	4.58	5.55	2.034	<0.001	0.063	0.382
Propionate/butyrate	0.34	0.24	0.16	2.68	1.79	0.86	0.139	<0.001	0.009	0.038
pH of fresh caecal content	6.54	–	–	6.45	–	–	0.078	0.558	–	–
pH of sample	6.79	6.53	6.38	6.37	5.98	5.71	0.066	<0.001	<0.001	0.581
Methane, mmol/kg	8.19	11.43	13.31	4.46	7.12	8.22	1.205	0.073	0.312	0.973
Gas release, mmol/kg	44.90	105.71	139.66	21.35	68.44	97.52	6.796	<0.001	<0.001	0.701

C control samples (incubated without the substrate); WB, OB the samples incubated with the substrate: wheat bran (WB) or oat bran (OB); ND not detected; mol% mol/100 mol of total SCFA concentration

Fig. 1 SCFA proportions in the rabbit and hare caecal content

requirements. In the cited research by Stott (2008), in which hares were heavier than rabbits, a proportionally higher caecal weight was also observed in the animals that weighed less. However, the development of digestive organs is dependent on diet, which was quite different in our animals that were caged from birth compared to that of the wild animals from captive collections used in the above-mentioned studies. Tao and Li (2006) reported a positive effect of the level of dietary NDF on caecum development in young rabbits; diets with higher NDF content significantly increased both the caecum weight and the caecum weight as a proportion of body weight with no effect on the absolute or relative weight of the caecal contents. On the other hand, it is possible that domesticated rabbits that have been offered rather high quality diets for many generations might develop proportionally smaller caecum compared to wild-type rabbits and hares.

Chemical analyses

Kuijper et al. (2004) found that the average diet of brown hares contained a higher NDF content compared to that of wild rabbits, and these differences were explained by the different feeding strategies connected to the different ecological niches of the two herbivores. However, their feeding strategies

mainly differed under unfavourable conditions when only low-quality forages were available. In this case, hares maximise food processing by increasing the intake rate and decreasing the digesta retention time, whereas rabbits maximise digestion by increasing the mean digesta retention time (Kuijper et al. 2004). In our experiment, the animals were offered the same diet and could not select their feed; they could only alter the proportion of pelleted feed or meadow hay consumed. Due to substantial differences in particular fibre fractions percentages between pellets and hay (Table 4), high contents of crude fibre, NDF and ADF in the stomachs indicate that both species consumed meadow hay in addition to the pelleted mixture. Since the chemical compositions of the gastric contents, including crude fibre, NDF and ADF, did not differ between both species, it could be supposed that hares consumed pellets and hay in comparable proportions to those consumed by rabbits. Thus, we suppose that when the available energy and nutrients are sufficient, rabbits and hares do not differ significantly in their selection of concentrate and bulk fodder. In our study, the similar digesta compositions allowed the activity of caecal microorganisms in both species to be compared.

Caecal fermentation profile

The pattern of microbial fermentation in the caecum is dependent on the composition and activity of the gastrointestinal microbiota. The colonisation of the rabbit caecum and colon by microbiota begins after birth and progresses toward a steady state from weaning, when the rabbit starts to consume solid feed, until approximately 70 days of age. The resulting bacterial populations are mainly represented by the *Bacteroides-Prevotella* and *Firmicutes* groups (Combes et al. 2011).

The microbial activity in the caecum determines the production of microbial metabolites in proportions characteristic of a particular species. In the rabbit caecum, acetate represents the highest proportion of the SCFAs followed by butyrate and then propionate, which results in a propionate/butyrate ratio of less than one (Bellier et al. 1995). The distinctive feature of the rabbit SCFA profile is that the molar proportion of butyrate exceeded that of propionate, what is opposite that in most herbivorous and omnivorous mammals, which produce more propionate than butyrate in their digestive tract (Bergman 1990). In our previous study (Mišta et al. 2015), lower SCFA production was found in the hare caecum compared to in the rabbit caecum, and it was explained by the lower digestibility of hemicelluloses in the hare (Stott 2008). In the present study, the total SCFA concentration was still lower in the hares regardless of the supplementary substrate, which was either rich in hemicelluloses (wheat bran) or rich in non-structural carbohydrates with a higher fat level (oat bran). Nevertheless, non-structural carbohydrates increased the total

SCFA concentration more than fibre in both species in our experiment, which was similar to the results of previous studies with rabbits (Belenguer et al. 2012). Zhu et al. (2015) showed that different dietary fibre/starch ratios significantly changed the composition of the caecal microbiota in growing rabbits, but diets with excessive fibre or starch reduced microbial diversity while more balanced diets containing moderate amounts of fibre and starch promoted a more diverse and abundant microbiota in the rabbit caecum.

The protozoa might interact with the bacteria, which is evident in ruminants and some hindgut fermenters such as the horse (Egan et al. 2010). It has also been confirmed that parasitic protozoa may affect the production of SCFA in the gut. Impairment of SCFA/HCO₃[−] exchange in the intestines was found during experimental rabbit coccidiosis (Manokas et al. 2000). In the present study, the number of oocysts in the faecal samples of both rabbits and hares was below the detection level. Such low invasion is common even in clinically healthy rabbits and hares (Połozowski 1993, Chroust et al. 2012), so it seems unlikely that it could influence SCFA production.

Different microbial activities in the caeca of rabbits and hares affected the SCFA profile of both species. Marounek et al. (2013) stated that the molar percentages of acetate and butyrate were lower and the molar percentage of propionate was higher in brown hares than in domestic rabbits. In our previous study (Mišta et al. 2015), no differences in the acetate molar proportion were found between species, but differences in the propionate/butyrate ratio were confirmed. The proportion of propionate in hares was higher, which affected the propionate/butyrate ratio, being approximately one, but that experiment was performed using captive hares that were only fed plants from the natural environment before they were caught, which constituted a different diet from the rabbit fodder used in that study. In the present study, in which both species were offered the same diet, the proportion of propionate in the control hare samples exceeded that of butyrate, contrary to the rabbit samples. Our current propionate/butyrate values in rabbits were below 0.5, which agreed with previous data for adult rabbits (Bellier et al. 1995; Garcia et al. 2002; Combes et al. 2011). In our hares, the propionate proportion was significantly higher whereas the butyrate proportion was significantly lower than in rabbits, which showed a reverse propionate/butyrate ratio (in our control group). Due to the higher level of propionate relative to butyrate, the SCFA profile in the hare caecum resembles the SCFA profiles of other mammals including ruminants, pigs, wild boars or coypus (Zawadzki 1993; Marounek et al. 2005; Suarez-Belloch et al. 2013; Pecka-Kiełb et al. 2016), and a similar SCFA proportion, with a higher level of propionate than butyrate, was also observed in young rabbits (Piattoni et al. 1996). However, in postweaned rabbits, the total SCFA concentration increased, and the proportions of acetate,

propionate and butyrate changed from approximately 80, 11 and 8 mol%, respectively, at 28 days of age to 75, 6.5 and 17 mol% at the 70th day (Combes et al. 2011), which were similar to our results for rabbits from the control group.

Further differences in the SCFA profile concerned the molar proportion of caproate, which did not exceed 0.5 mol% in rabbits, but its proportion was more than tenfold lower in hares. In the hare control samples, caproate was below the detection limit, which was also reported in the caecum of another herbivorous mammal, the coypu (Marounek et al. 2005).

The addition of a supplementary substrate to the incubated samples, especially oat bran, mainly increased butyrate, which was reflected by lower propionate/butyrate ratios in both species. The use of wheat bran, a fibre-rich substrate, increased butyrate in rabbits, but the proportion of propionate was not changed. Similarly, only a very small decrease in the level of propionate was observed in hares. More changes were caused by the use of the substrate rich in NSC and fat, i.e. oat bran, which increased butyrate and decreased propionate in both species compared to the control group. Our results, which showed a decrease in the proportion of acetate and an increase in the proportion of butyrate after the addition of oat bran, correspond with Garcia et al. (2002), who found that the proportion of acetate tends to increase while butyrate tends to decrease with an increased in the content of dietary NDF.

In our experiment, the structural fibre in bran is mainly represented by hemicelluloses (approximately 29 and 10% of DM in wheat and oat bran, respectively), which are important components of feeds for rabbits as well as important substrates for intestinal microbes (Gidenne et al. 2000, 2002; Gidenne and Fortun-Lamothe 2002; Lavrenčič 2007). The starch in wheat and oat bran is digested in the small intestine, but some of it reaches the rabbit caecum where it is fermented by microbiota. In our study, starch-rich oat bran fermented by the caecal microbiota caused greater SCFA and gas release than wheat bran, which is richer in hemicelluloses; this is in contrast with the finding of Lavrenčič (2007), who reported a similar fermentation rate for hemicelluloses and starch incubated with caecal inoculums of 78-day-old rabbits. To our surprise, changes in the pH of incubated samples did not correlate with the SCFA concentration; the samples with hare caecal inoculums had lower pH values than the corresponding rabbit caecal samples despite the lower SCFA concentration in hares. It is noteworthy that the pH values of fresh caecal digesta were similar in both species.

Methanogenesis

In adult rabbits, methanogenic Archaea, which reduce carbon dioxide to methane, are known to make an essential contribution to the caecal microbiota, representing up to 22% of the total microbial RNA (Bennegadi et al. 2003). The presence of

Archaea was not confirmed in rabbits on the 2nd day after birth, but methane production began on the 7th day and increased up to the 70th day (Combes et al. 2011).

In the present study, the tendency toward lower methane production in incubated hare caecal samples compared to rabbit samples could be explained by the sensitivity of methanogenic Archaea to low pH. Previous research conducted in ruminants showed a considerable decrease in methanogenesis when the pH dropped below 6 (Russell 1998), and our hare samples that were incubated with supplementary substrate had a final pH below this value. Nevertheless, in our control hare samples, which contained diluted caecal contents without any additional substrate, methanogenesis was lower compared to rabbits despite the pH values being above 6. Moreover, our previous research showed much lower methanogenesis in wild hares (below 0.3 mmol/kg of caecal content) compared to rabbits (above 11 mmol/kg) despite non-significant differences in caecal pH between species (Marounek et al. 2013; Mišta et al. 2015). The low methanogenesis was not caused by a lack of methanogens because a low level of methane was always released from the hare caecal cultures in those experiments. In the present study, a large variation in methanogenesis was observed among animals of the same species, which resulted in no statistically significant differences. For example, in six hares and one rabbit, methane was produced in very small amounts, below 1 mmol/kg and sometimes even below the detection limit, while in 3 hares and 4 rabbits, methanogenesis exceeded 14 mmol/kg. It could be supposed that although methanogens show very low activity in wild hares (probably due to unfavourable conditions and a shortage of hydrogen in their digestive tract), they could begin to proliferate and produce more methane in response to a change in the diet of the animal. However, their growth is not only determined by diet but also depends on other factors, including the conditions of the digestive tract of individual animals. For rabbits, Marounek et al. (1999) also found a lack of methanogenesis in 1 of 11 caecal samples incubated *in vitro*. Belenguer et al. (2011) found that only 2 out of 16 rabbits produced a substantial volume of methane *in vivo*, but an *in vitro* study of these authors showed methane production in all samples. Individual differences in methane production in the digestive tract also exist in other species, including humans, where methane is consistently excreted in an appreciable quantity by some subjects but not others (Strocchi et al. 1991). Numerous investigators have tried to find a reason for these differences in the level of methanogenesis. Šustr et al. (2014), who used millipedes as a model to determine the factors influencing gut methanogenesis, reported that variability in CH₄ production among millipedes reflected differences in the activity and proliferation of methanogenic Archaea in the digestive tracts of some phylogenetic lineages rather than a fundamental inability of these lineages to host methanogens. Our studies in leporids support that statement because methane was always released, although in varying levels, from the caecal cultures of our animals, which is

evidence of the presence of methanogens. Low emission of methane despite the presence of methanogenic Archaea in the digestive tract was stated also in other animals, e.g. Australian macropodids (Hoedt et al. 2016). Recent studies reported similar abundance of methanogens communities in rumen of sheep being considered high methane producers to that in rumen of sheep being low methane producers (Shi et al. 2014). The feature of low and high methane emission in animals of the same species appeared heritable which allowed the authors of that study to select animals for a phenotype producing low amount of methane. The authors found that the level of methanogenesis depended on the expression levels of the hydrogenotrophic methanogenesis pathways in rumen methanogens rather than on methanogens abundance. In low methane producers, there appears a reduction in the expression of genes responsible for the hydrogenotrophic methanogenesis pathway which may be a response of the resident methanogens to changing of environmental conditions, such as the hydrogen supply (Shi et al. 2014). Hoedt et al. (2016) found that alcohol-fueled methanogenesis existing in herbivores with low methane emission may be an important evolutionary adaptation of methanogens to persist in the unfavourable intestine environment.

Methanogens that inhabit human and animal digestive tracts use substrates originating from the anaerobic degradation of organic matter by anaerobic bacteria (Gaci et al. 2014), and most are hydrogenotrophs that use hydrogen to reduce carbon dioxide to methane (Liu and Whitman 2008). Hydrogen, together with carbon dioxide production, accompanies the microbial fermentation of carbohydrates to SCFA. Methanogenic Archaea must compete to uptake metabolic hydrogen with acetogenic and sulphate-reducing bacteria in the large intestine. The substrate affinity of these microbes for hydrogen seems to be ambiguous and to depend on several factors. According to Gibson et al. (1990), the sulphate concentration in the large intestine is critical for determining which of these processes occurs, and if there is sufficient sulphate availability, sulphate-reducing bacteria will predominate. However, Strocchi et al. (1991) stated that methane-producing bacteria outcompete other H₂-consuming bacteria for H₂ in human faeces despite sufficient sulphate availability. The acetogenic bacteria, which reduce carbon dioxide to acetate, were only active when the activity of Archaea and sulphate-reducing bacteria was low (Gibson et al. 1990), and in rabbits, reductive acetogenesis is partly replaced by methanogenesis with age as young animals began to intake solid food (Piattoni et al. 1996). Abecia et al. (2013) observed relatively low methanogenic Archaea abundance in the rabbit caecum compared to the goat rumen, which suggested that methanogenesis may not be the major hydrogen sink in rabbit caecal ecosystems.

Intestinal pH was found to be one of the main factors limiting the microbial competition for hydrogen because microbial populations vary in their sensitivity to low pH. When pH

is close to 6.5, the growth of acetogenic bacteria was most favoured, whereas a pH close to 7 supported the growth of methanogens; pH 7.5 was the most preferred by sulphate-reducing bacteria (Gibson et al. 1990). Surprisingly, based on the analysis of individual differences in methane production of the animal samples in the present study, it could be stated that caecal pH was not the only factor affecting methanogenesis because the samples that showed very low methane production had different pH values (from 5.07 to 6.91). This observation supported our earlier assumption that the differences in methane levels between rabbits and hares were not only caused by caecal pH but other factors, such as phylogenetic and genetic, as well (Mišta et al. 2015).

The substrates used in this study, especially the oat bran, caused an increase in methane emission, visible only in individuals of both species, but due to the variability among the individual animals, we were not able to detect any significant differences. The increase in methane production due to substrate addition was accompanied by an increase in the total gas and SCFA production in incubated samples. Low hydrogen availability limits methanogenesis (Strocchi et al. 1991). Therefore, the level of methane production is related to the activity of fermentative bacteria, which increase the partial pressure of H_2 in the caecum (Marounek et al. 1999; Belenguer et al. 2011). It could be supposed that the availability of the substrate made the caecal environment more favourable for methanogenic Archaea, but methanogenic activity could also be modulated by other factors connected with the intestinal environment, such as different sources of starch in the feed. Belenguer et al. (2011) observed greater methane formation in rabbit caecal inocula when maize was used as the source of starch instead of wheat. The problem of the variability in methane production in the caecal inocula of leporids observed in our studies is probably multi-faceted and connected with several factors related to the intestinal ecosystem as well as genetic effects. Nevertheless, comparing the present results with our previous studies on wild hares, it could be assumed that feeding commercial feed to hares induced caecal methanogenesis in some cases, although the levels were highly variable among individual animals.

Conclusion

Our study showed lower microbial activity in the caecum of brown hares compared to domestic rabbits, which was manifested as a lower total SCFA concentration and lower release of gases by caecal microbiota. Hares and rabbits, despite being offered the same diet, exhibited different microbial fermentation patterns. The propionate/butyrate ratio in hares was close to one, while the molar proportion of butyrate exceeded that of propionate more than fourfold in rabbits. Methanogenesis tended to be lower in hares than in rabbits, but high individual

variability was observed, especially in hares. Thus, the commercial feed offered to the hares led to an increase in the activity of caecal methanogens and caused the production of a substantial amount of methane, but not in all animals. Further research focused on profiling and identifying the caecal microbiota in the brown hare could explain individual differences in the activity of methanogens in this species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the Wrocław University of Environmental and Life Sciences where the studies were conducted. This experiment was carried out with the approval from the Local Ethics Commission for Experiments on Animals in Wrocław, Poland (Licence No. 46/2015).

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