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In Vitro Study of Caecal and Colon Microbial Fermentation Patterns in Wild Boar (*Sus scrofa scrofa*)*

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The aim of this study was to evaluate wild boar (*Sus scrofa scrofa*) caecal and colon products of microbial activity including short chain fatty acids (SCFA), ammonia and methane concentrations. The *in vitro* method was applied to caecal and colon contents after 12 and 24-hour incubation with the substrate (wheat bran), or without any additive (control samples). The pH was also measured in each sample. In samples incubated with the substrate, a lower pH was noted as compared to the control ($P<0.001$). In terms of the total SCFA concentration, the hindgut microbial fermentation pattern of wild boar was characterized by a high acetate level, followed by propionate and then butyrate at a ratio of 7:1.5:1. Substrate addition decreased acetate molar proportions ($P<0.001$) and increased those of butyrate ($P<0.001$) as well as propionate ($P<0.05$). The total SCFA level in fresh, unincubated caecal samples (128 mmol/kg) was similar to that in the colon (111 mmol/kg). The ammonia concentrations were at the level of 0.8-1.5 mmol/kg of hindgut content and did not differ between the two investigated hindgut parts. Methanogenesis was also similar in the caecum and colon and after 24h was 2.69 mmol/kg and 2.27 for caecal colon control samples, respectively. The substrate increased total gas production and methane concentration ($P<0.001$).

Key words: Wild boar, fermentation *in vitro*, hindgut, short chain fatty acids, ammonia, methane.

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Microbial fermentation in the gastrointestinal tract (GIT) of animals is a species-specific physiological adaptation and depends primarily on the size of the animal and its food habits (PAGAN 2011). Digestive enzymes produced by microorganisms allow for the digestion of food ingredients which would otherwise not be utilized (BAROWICZ 2011). In recent years, the microbial fermentation of non-starch polysaccharides or dietary fibre in the GIT of monogastric animals has been of considerable interest (MAROUNEK *et al*. 1997, 2002; MIKKELSEN *et al*. 2004; VAN NEVEL *et al*. 2006). The most important fermentation chambers in monogastric animals, including wild boar (*Sus scrofa scrofa*), are the caecum and colon. The concentrations of the end products of bacterial fermentation such as short chain fatty acids (SCFA), ammonia and gases including H_2, CH_4 and CO_2, reflects the activity of the intestinal microflora which determines the correct course of the processes in the GIT (FORTUN-LAMOTHE & BOULLIER 2004). SCFA are physiologically important, especially in the large intestine in which butyrate in particular is required to maintain the health of epithelial cells lining the gut (ROEDIGER 1980; JENSEN & JØRGENSEN 1994).

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The wild boar population in Poland is vast. It is necessary to control the population by means of effective hunting, based on an accurately conducted inventory of animals (KEULING *et al.* 2013). Nevertheless, physiological processes in the gastrointestinal tract of these animals have not been precisely studied. Although wild boars prefer high-protein food, compared to the domestic pig (*Sus scrofa domestica*) they have a better developed large intestine which could indicate a considerable contribution of this segment of the GIT in the digestion of carbohydrates (LEROCH 2001). However, there are no published data concerning microbial activity occurring in the wild boar hindgut, whereas many authors have described these physiological aspects in the boar's domesticated relative – the pig (WANG *et al*. 2004; LOH *et al.* 2006; VAN NEVEL *et al.* 2006; SUAREZ-BELLOCH *et al.* 2013).

Microbial fermentation can be estimated using an *in vitro* batch culture technique. The aim of this study was to evaluate caecal and colon products of the microbial activity of wild boars living in their natural habitat.

Material and Methods

Study area and animals

The study area was located in hunting circuit No. 232 Łęknica, Lubuskie province, western Poland. Nine wild boars (*Sus scrofa* (L.)) – three females and six males aged 1-2 years with an average body weight of 35 kg – were used in the study. Samples were collected from wild boars hunted in November 2012. The hunts took place between 10 and 12 a.m. Subsequently, the GIT of each wild boar was removed from the body and brought to the laboratory. The caecum and colon were excised and slit. Then, digesta were transferred to separate containers and transported to the laboratory in 39°C and anaerobic conditions. The caecal and colon contents were used as inoculum for *in vitro* incubation.

In vitro fermentation model

In order to prepare samples for *in vitro* fermentation, 20 g of the contents of caecum and colon, respectively, were diluted in 60 ml of buffer (pH 7.2) (ADJIRI *et al.* 1992) and transferred into serum bottles with a capacity of 125 ml (Sigma-Aldrich). These solutions were used as inoculum samples for *in vitro* incubation. The samples were divided into two groups: C (control), in which the intestinal contents were incubated without any addition and group S (substrate), in which 1 g of wheat bran was added to the contents. In order to obtain anaerobic conditions, serum bottles were flushed with CO_2 from a pressure bottle and immediately afterwards closed tightly with a manual crimper. The samples were incubated in a shaking water bath at 39°C for 12 and 24 hours.

pH measurement, SCFA and ammonia analyses

Immediately after the caecal and colon digesta were received, the pH in the obtained samples was measured using a CP-401 pH-meter (Elmetron, Zabrze, Poland) with an EPP-3 electrode and temperature sensor. The pH was also measured in the incubated and unincubated samples of caecum and colon diluted with buffer (pH 7.2).

In the unincubated samples and after 12 and 24 hours of incubation, measurements were taken of the total SCFA concentration and the percentage of each fatty acid: acetate, propionate, isobutyrate, butyrate, isovalerate and caproate. At the end of incubation, fermentation was stopped by adding formic acid in order to inhibit fermentation processes. The SCFA concentrations were analysed using a gas chromatograph 7890A (Agilent Technologies, Palo Alto, USA) with a flame ionization detector (FID) and Agilent J&W DB-23 column, with helium as a carrier gas.

In the same samples, the ammonia level was determined using the modified microdiffusion method of Conway with a Nessler reagent, and estimated by spectrophotometry (Perkin Elmer Lambda XLS spectrophotometer) at wavelength $\lambda = 410$ nm.

Gas production and methane analysis

After 12 and 24 hours of incubation, the fermentation gas was sampled for analysis with a gas-tight syringe. Additionally, before the gas analysis, the headspace pressure inside each bottle was measured. Then, in the remaining liquid sample, fermentation was stopped by adding formic acid. The amount of methane was determined using a gas chromatograph 7890A (Agilent Technologies) with a thermal conductivity detector (TCD) and FID.

Statistical analysis

The data concerning the incubated samples were subjected to a three-way ANOVA using the STATISTICA PL 10. software package. Three main effects – A, B, and C were tested: A – the effect of the hindgut part (caecum or colon), B – the effect of the substrate (with or without) and C – the effect of the incubation time (12 or 24 h); in addition, three interactions were analysed: A x B, A x C, and B x C. The data obtained from analyses of un-

incubated samples were subjected to a one-way ANOVA (STATISTICA PL 10), where the effect of the hindgut part was tested. Significance was declared at P<0.05. Differences between means with 0.05<P<0.10 were interpreted as tendencies.

Results

The pH values, SCFA and ammonia concentrations of unincubated hindgut samples are presented in Table 1. The pH of fresh caecal and colon content occurred at similar levels and averaged 5.73±0.41 and 5.74±0.38, respectively. Mixed with buffer, unincubated caecal samples were characterized by a pH of 6.84 and the pH of colon samples was 6.96 (Table 1). In the samples incubated with the substrate, a lower pH was noted as compared to the control (P<0.001) (Table 2). A decreasing tendency for pH was also observed when the incubation time was prolonged up to 24h.

An increase in SCFA concentration in the hindgut samples was observed as the effect of wheat bran addition (P<0.01) (Table 2). There were no differences between caecal and colon SCFA concentrations in either unincubated or incubated

Table 1

Caecal and colon parameters of unincubated samples

Hindgut parameters	Caecum	Colon	S.E.M.	P value
pH	6.84	6.96	0.069	0.439
SCFA [a]	128.06	111.35	10.56	0.448
Acetate [b]	73.21	70.40	0.78	0.069
Propionate [b]	10.57	12.67	0.59	0.073
Isobutyrate [b]	0.36	0.48	0.10	0.581
Butyrate [b]	10.05	10.73	0.42	0.443
Isocaproate [b]	0.05	0.52	0.18	0.206
Caproate [b]	5.33	4.72	0.36	0.417
Hexanoate [b]	0.32	0.35	0.05	0.727
Heptanoate [b]	0.11	0.14	0.03	0.697
Ammonia [a]	1.47	0.94	0.15	0.070

[a] mmol/kg of intestinal content;
[b] percentage in the total molar concentration of SCFA (mol %)

Table 2

Caecal parameters after the 12 and 24h of *in vitro* fermentation

Fermentation parameters	CAECUM				COLON				S.E.M.	P value		
	12h		24h		12h		24h			A	B	C
	C	S	C	S	C	S	C	S				
pH	6.33	5.95	6.18	5.77	6.17	5.83	6.08	5.69	0.04	0.111	<0.001	0.059
SCFA [a]	282.43	344.58	308.99	417.09	312.66	364.77	305.63	359.00	12.28	0.911	0.007	0.383
Acetate [b]	69.57	64.72	67.68	62.03	70.62	62.94	63.80	57.09	0.83	0.087	<0.001	0.003
Propionate [b]	13.28	15.82	14.32	15.54	13.98	16.10	15.80	18.31	0.41	0.107	0.011	0.138
Isobutyrate [b]	0.70	0.43	0.66	0.76	0.56	0.43	0.64	0.64	0.05	0.508	0.475	0.164
Butyrate [b]	10.66	13.14	11.09	15.23	9.35	14.27	13.06	16.81	0.43	0.205	<0.001	0.002
Isocaproate [b]	0.90	0.94	1.09	1.33	0.72	0.72	0.94	1.08	0.08	0.237	0.528	0.087
Caproate [b]	3.91	3.67	3.96	3.71	4.26	4.31	4.49	4.60	0.20	0.154	0.848	0.711
Hexanoate [b]	0.75	0.92	0.83	1.04	0.36	0.91	0.88	1.05	0.08	0.594	0.080	0.175
Heptanoate [b]	0.24	0.36	0.37	0.36	0.16	0.31	0.39	0.41	0.03	0.866	0.241	0.092
Gas production [a]	82.37	150.67	112.76	179.28	109.12	153.48	125.13	191.53	5.69	0.090	<0.001	<0.001
Methane [a]	2.11	3.54	2.69	3.91	1.78	2.97	2.27	4.67	0.21	0.702	<0.001	0.037
Ammonia [a]	0.97	0.89	1.21	1.14	1.37	0.82	1.04	1.09	0.07	0.902	0.245	0.426

A – effect of part of the hindgut, B – effect of the substrate, C- effect of the incubation time
[a] mmol/kg of undiluted intestinal content;
[b] percentage in the total molar concentration of SCFA (mol %)

samples. The highest SCFA level was found in caecal samples incubated for 24h with the substrate (419.09 mmol/kg).

In the SCFA profile of the wild boar, acetate levels were the highest (about 72% for unincubated and 65% for incubated samples), followed by propionate (12 and 15%, respectively) and then butyrate (10 and 13%, respectively) in terms of the total SCFA concentration of hindgut. Additionally, no significant differences in this profile were noted between caecal and colon samples (Tables 1 and 2). However, tendencies for lower acetate and higher propionate molar proportions for unincubated colon samples were observed as compared to the caecal ones (Table 1). The tendency for lower acetate in the colon content was confirmed when incubated samples were analysed (Table 2). The substrate addition decreased acetate ($P<0.001$) and increased butyrate ($P<0.001$) as well as propionate molar proportions ($P<0.05$) (Table 2). Moreover, we also noted an increasing tendency for caproate molar proportions as the effect of substrate addition to the hindgut contents. Acetate decreased, butyrate increased ($P<0.01$) and isovalerate as well as caproate tended to increase when the fermentation time was prolonged from 12 to 24h ($P<0.01$) (Table 2).

The ammonia concentrations in the analysed caecal and colon samples were maintained at similar levels after 12 and 24 fermentation hours. In the unincubated samples, the ammonia levels tended to be higher for caecal (1.47 mmol/kg) than for colon content (0.94 mmol/kg).

The gas production during colon fermentation tended to be higher than caecal gas production and significantly increased after substrate addition, as well as when the incubation time was prolonged ($P<0.001$) (Table 2). Similarly, substrate ($P<0.001$) and time ($P<0.05$) effects of methane production for the analysed hindgut samples were also found. The highest methane level was achieved after 24h for samples incubated with substrate addition, and amounted to 3.91 and 4.67 mmol/kg for the caecal and colon content, respectively.

Insignificant differences were found when the interactions between the effects of the part of the hindgut (caecum or colon), substrate (with or without) and incubation time (12 or 24h) were tested (not shown).

Discussion

Wild boars are omnivores: approximately 70% and 30% of their diet consists of plant and animal food, respectively. However, the proportions of the ingested food types depend on many factors including geographic location, regional food availability and the age of the animal (SCHLEY & ROPER 2003). Favourite ingredients in the diet of wild boars include corn, beech nuts, berries and other fruits of the forest, as well as grass, herbs, shoots of trees and shrubs. Food of animal origin eaten by boars consists of the eggs and chicks of nesting birds, frogs and young mammals. They also eat carrion found on their territory (PILARCZYK *et al.* 2010). The diet of the animals used in the present study was composed of seasonally available food, mainly corn cobs and acorns.

The most intense microbial fermentation processes occur in monogastric mammals in the caecum and colon. Undigested foods are metabolized by local microflora. Since there is a lack of data concerning the activity of the hindgut microflora of wild boar, we discussed our results with reference to its domesticated relative – the domestic pig.

In comparison to the results obtained for the large intestine of the domestic pig, the active acidity (pH) of fresh content in the large intestine of wild boar was at a slightly lower level. The pH of the caecal contents of an adult pig depends on the diet and varies from about 6.03 (MAROUNEK *et al.* 2002) to 6.7 (EBERHARD *et al.* 2007), whereas in wild boars in the present research caecal pH achieved a value of 5.73. In the pig colon, pH is slightly higher and varies from 6.2 (GOVERS *et al.* 1999) up to 7.1 (LOH *et al.* 2006), whereas colon pH of wild boars did not differ from that of the caecum and was about 5.74. According to JENSEN & JØRGENSEN (1994), pH in the pig caecum depends on the fibre content of the diet. These authors noted lower pH values for caecum and colon in pigs receiving a high-fibre diet (5.6 and 6.0, respectively), than values in pigs receiving a low-fibre diet (6.3 and 6.5, respectively). Based on these results, we suggest that the lower pH in the wild boar large intestine is also caused by the high-fibre diet of this animal. Pigs fed a diet with wheat bran have lower caecal and colon pH (6.1 and 6.2, respectively) than animals fed a control diet (6.3 in both hindgut parts) (GOVERS *et al.* 1999). Our results confirmed the observations that the addition of wheat bran decreased the hindgut pH.

In unincubated samples from the caecum and colon of wild boars, the level of SCFA production was similar to that of domestic swine, in which it is highly correlated with diet and ranges widely from 65.3 mmol/l for caecum (SUAREZ-BELLOCH *et al.* 2013) and 77.2 mmol/l for colon (LOH *et al.* 2006) up to 125.8 and 149.4 mmol/l for caecum and colon, respectively (VAN NEVEL *et al.* 2006). In our experiment, the addition of wheat bran as a substrate increased the production of SCFA in the caecal and colon contents, whereas wheat bran addition to the pig diet did not change total SCFA

concentration in the caecum and colon, but increased it in the faeces (GOVERS *et al*. 1999). Similarly, VAN NEVEL *et al*. (2006) did not find differences in pig caecal and colon SCFA concentration after wheat bran addition during in vivo experiments.

Our data indicated that SCFA patterns in the caecum and colon of wild boar differ from those observed in pigs and are characterized by a lower molar proportion of propionate. The pig caecal SCFA profile is characterized by 53-64 acetate, 22-25 propionate and 10-23 butyrate molar proportion (MAROUNEK *et al*. 1997; VAN NEVEL *et al*. 2006; SUAREZ-BELLOCH *et al*. 2013). This profile is similar in the colon, and consists of 54-65% acetate, 21-35% propionate and 7-14% butyrate (LOH *et al*. 2006; VAN NEVEL *et al*. 2006; VON HEIMENDAHL *et al*. 2010; LIZARDO *et al*. 2012). According to VAN NEVEL *et al*. 2006, the caecal SCFA profile of pigs is similar to that of the colon. Our results concerning wild boars show some tendencies for lower acetate and higher propionate molar proportions for colon samples as compared to the caecal ones. According to WANG *et al*. (2004), wheat bran addition increased the molar proportions of acetate and propionate, whereas it lowered butyrate after 48-hours incubation of fresh rectal faeces. In wild boar hindgut, we observed an increase in propionate and butyrate, accompanied by a decrease in acetate levels.

SCFA produced in the hindgut are an important energy source for animals and contribute about 25% of the energy source for pigs (BERGMAN 1990). Moreover, propionate is a very effective substrate for glucose synthesis, and participates in glycogenesis and the formation of long-chain fatty acids in the liver. Acetate participates in lipogenesis, milk fat synthesis, cholesterogenesis and ketogenesis (BERGMAN 1990; RÉMÉSY *et al*. 1995). Butyrate is the main energy source for intestinal epithelial cells, and an increased level improves the health status of the gut (BINDELLE *et al*. 2008).

The iso-acid proportions in the caecum of the domestic pig show a lower ratio of isobutyrate to isovalerate (MAROUNEK *et al*. 2002; SUAREZ-BELLOCH *et al*. 2013). We observed a similar relationship in wild boars. The level of iso-acids in the caecum of wild boar ranged from 0.41 to 2.09 mol%, while this range in pig caecum is higher from 3.2 to 9.5 mol% (MAROUNEK *et al*. 2002) and from 2.5 to 3.0 (SUAREZ-BELLOCH *et al*. 2013). Cellulolytic bacteria utilize branched-chain SCFA for their proliferation, while ammonia is the main source of nitrogen for the fibrolytic bacteria, which have the ability to synthesize cellulolytic and hemicellulolytic enzymes (MAROUNEK *et al*. 2002).

The presence of ammonia and iso-acids in the caecum and colon indicate the proteolytic activity of the local microflora. According to O'SHEA *et al*. (2011), ammonia concentrations in the caecum of pigs are about 1.89 mmol/l, and in the colon approximately 4.08 mmol/l. A lower ammonia level was found in the analysed samples from the colon contents of wild boar. The application of wheat bran in domestic swine diet lowered the levels of ammonia in the content of the large intestine (VAN NEVEL *et al*. 2006). In wild boars, when wheat bran was used as a substrate for in vitro fermentation, no differences were found in terms of ammonia production.

The bacterial fermentation of carbohydrates in the hindgut is accompanied by gas production, including methane. Methanogenesis is higher in pig colon than in the caecum and increased when pigs were fed high-fibre diets (JENSEN & JØRGENSEN 1994), whereas our results showed no differences in wild boar between colon and caecal methanogenesis. Methane production in pigs is substantial and after 8 hours of the fermentation of caecal content reaches 4.14 mmol/l (MAROUNEK *et al*. 1997). Our study showed that methanogenesis in wild boar was lower. After 24 h of pig caecal fermentation, the methane percentage in the total gas volume was about 10.3 % (GONG *et al*. 2013), whereas in the present study the proportion of methane was only about 1 % of total gas volume. It has been stated that pig colon methanogenesis is larger than that of the caecum (LAERKE *et al*. 2000). In the wild boar, no differences in methane production were found between hindgut parts.

In conclusion, in the large bowel of wild boar, the microbial fermentation pattern shows highest levels of acetate, followed by propionate and then butyrate in terms of the total SCFA concentrations, i.e. in relative proportions about 7:1.5:1. The addition of wheat bran as a substrate in microbial fermentation decreased acetate, but increased butyrate and propionate ratios. The microbial populations colonizing the hindgut show methanogenic activity, although lower methane production was observed in wild boars compared to domestic pigs according to previous studies. The substrate we used increased total gas production and methane concentration. Moreover, the presence of ammonia and iso-acids in wild boar caecum and colon indicate the proteolytic activity of the microflora. Due to the limited data concerning microbial activity in wild boar hindgut, further studies are needed to extend our knowledge of this topic.

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