SUPPLEMENTATION OF RABBIT DIET WITH CHESTNUT WOOD EXTRACT: EFFECT ON *IN VITRO* GAS PRODUCTION FROM THREE SOURCES OF CARBOHYDRATES

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ABSTRACT

In vitro gas production kinetics of barley grain (BG), sugar beet pulp (SBP) and wheat bran (WB) were determined using three different inocula prepared from caecum contents of 78 day old rabbits. The first inoculum was prepared from caecum contents of rabbits fed diet supplemented with 0.5% of chestnut wood extract (CWE, 75% of tannins) in the form of powder (POWD), the second one from caecum contents of rabbits which diet were supplemented with 0.6% of coated CWE (COAT), while the third one from caecum contents of rabbits not supplemented with CWE (CONT). The cumulated gas productions over 60 h of incubation were modelled with Gompertz model. The total potential gas productions (parameter "B" of the Gompertz model) were higher when BG and SBP were incubated in CONT (300 and 288 ml for BG and SBP, respectively) than in COAT (270 and 264 ml for BG and SBP, respectively) and POWD (278 and 264 ml for BG and SBP, respectively). For WB the highest parameter "B" was obtained when incubated in POWD; however, the differences between inocula were not statistically significant ($P>0.05$). Maximum fermentation rates (MFR) of BG (21.1 ml/h) and WB (9.7 ml/h) incubated in CONT were greater than those incubated in POWD (16.3 and 8.7 ml/h for BG and WB) or COAT (18.5 and 8.1 ml/h for BG and WB). However, for SBP the greatest MFR was obtained when incubated in POWD (15.1 ml/h), followed by CONT (13.3 ml/h) and COAT (11.8 ml/h). The times of maximum fermentation rate (TMFR) were generally shorter for substrates incubated in POWD (12.6, 7.4 and 8.4 h for BG, SBP and WB, respectively) than those incubated in CONT and COAT (12.9 and 12.9 h for BG, 9.5 and 9.4 ml for SBP and 9.5 and 8.4 ml for WB, respectively). In the first 8 h of fermentation (Gas8), higher ($P<0.05$) amounts of gas were produced from substrates incubated in POWD (106, 60 and 35 ml for SBP, WB and BG, respectively) than in CONT (88, 46 and 23 ml for SBP, WB and BG, respectively) and COAT (78, 48 and 23 ml for SBP, WB and BG, respectively). In conclusion, the use of CWE as powder increases the microbial activity of caecal microorganisms and that the effect is greater with pectinolytic than with amylolytic or hemicellulolytic microbes and/or enzymes. The similar TMFRs and Gas8s of substrates incubated in COAT and CONT suggest that the process of coating was not very efficient as the CWE was not released at the beginning (in first hours) of incubation in the rabbit caecum contents.

Keywords: Rabbits, *In vitro* gas production, Carbohydrates, Barley, Sugar beet pulp, Wheat bran.

INTRODUCTION

Man and animals live in symbiosis with (intestinal) microbes. Species of intestinal flora colonizing the intestine could have either beneficial or harmful effects on animal health and productivity. To control the activity of microbes, especially the harmful ones, until recently the nutritive antibiotics were used. However, from the January 2006 the use of nutritive antibiotics in EU has been banned and much research effort is oriented toward disclosure of natural substances with similar properties. Among these (poly) phenolics, especially tannins could have great potential.

The main characteristic of tannins is their ability to form complexes with proteins, amino acids, polysaccharides, metal ions, vitamins, bacterial cell membranes and enzymes involved in protein and

carbohydrate digestion (Makkar, 2003). The complexes between tannins and microbial enzymes and between tannins and nutrients could limit the availability of the substrates to intestinal microorganisms, while the binding of tannins to cell membranes has a direct effect on the activity of microbes (Butter *et al.*, 1999; McSweeney *et al.*, 2001).

Fermentative activity of caecum microorganisms could be estimated using the *in vitro* gas production technique. The technique is carried out under strictly anaerobic conditions and is being used to examine the activity of the microflora from many different sources, including the rumen (Menke and Steingass, 1988; Lavrenčič and Stefanon, 2001) and GIT and faeces of pigs (Bauer *et al.*, 2004; Bindelle *et al.*, 2007), and caecum contents of rabbits (Calabrò *et al.*, 1999; Lavrenčič, 2007). Even if the processes of digestion occurring *in vitro* will never be identical to those occurring *in vivo*, the gas production technique could still be used to characterise feed fermentation in the large intestine and caecum of non-ruminant animals (Bauer *et al.*, 2004; Bindelle *et al.*, 2007).

The aim of the study was to evaluate the supplementation of rabbit diets with two forms of chestnut wood extracts (CWE offered as powder and coated with plant oils) on *in vitro* gas production kinetics of three different energy (carbohydrate) sources, usually used in rabbit diets: barley, sugar beet pulp and wheat bran. The desired effect of coated CWE should be obtained in the large intestine and caecum, where the tannins should be released and act against harmful microbes. On the contrary, nonprotected tannins (fed as powder) form complexes with substrates (and digestive enzymes) in the upper GIT, thus limiting the utilization of nutrients and impairing the productivity of animals.

MATERIALS AND METHODS

Substrates

Three different raw feeds, barley grain (BG), sugar beet pulp (SBP) and wheat bran (WB) were chosen because they are commonly used feed in an average rabbit diet and because they differ in the carbohydrate composition. The prevailing nutrients in these feeds are carbohydrates, starch in BG, pectins in SBP and hemicelluloses (presumably xylans) in WB (Kersten *et al.*, 2005). All substrates were milled to pass 1 mm screen before the *in vitro* trial.

In vitro fermentation

Manipulations and selection of animals and the preparation of inoculum were performed according to the methods described by Calabrò *et al.* (1999) and Lavrenčič (2007). Two New Zealand White rabbits (Slovenian meat line SIKA) were fed the commercial compound feed (Krka, Novo mesto, Slovenia; CONT), two animals were fed the same compound feed supplemented with 0.5% of CWE powder (POWD; commercial product Farmatan®, 75% of tannins; Tanin, Sevnica, Slovenia), while two animals received the compound feed supplemented with 0.6% of CWE (Tanin, Sevnica, Slovenia) coated (COAT) with plant oils (Polaris, France). Diets were offered *ad libitum* from weaning at 35 days of age. The animals were sacrificed at 78 days of age and the caeca were isolated by tying off the two extremities with nylon string to prevent movement of the digesta. The inocula were prepared mixing together the two caecum contents of animals fed CONT, POWD or COAT compound feed. Further manipulations were performed according to Lavrenčič (2007).

Calculations and statistical analysis

The gas produced at different times of incubation was corrected for the amount of gas produced from blank samples at the corresponding times within each repetition and type of inoculum. Obtained values were also corrected for the dry matter (DM) contents of samples. Corrected values were then fitted to the Gompertz model (Lavrenčič *et al.*, 1997). Parameter values and curve fitting were estimated by the Marquard compromise of a non-linear regression method, using SAS software (Proc NLIN) (SAS, 1994). From the estimated parameters of Gompertz model other parameters were

calculated: maximum fermentation rate (MFR; ml/h), time of maximum fermentation rate (TMFR; h), delay in fermentation at the start of incubation (LAG; hours) and volume of gas produced until 8 hours of incubation (Gas8). Data concerning fermentation kinetic parameters (parameters B, C, A, LAG, MFR, TMFR and Gas8) were tested for significance by analysis of variance using the Scheffe test (SAS, 1994) with the model where substrate and type of inoculum were fixed effects.

RESULTS AND DISCUSSION

The estimated parameters of *in vitro* gas production of the various substrates are shown in Table 1 where the main effects of substrate and type of inoculum are also presented. The estimated fermentation kinetic parameters of different substrates were significantly different ($P<0.001$). There were also significant differences in fermentation kinetic parameters ($P<0.05$) according to the type of inoculum, except in parameter A. Furthermore, there were significant interactions for kinetic parameters C and A of Gompertz model.

Within substrates the total potential gas production (parameter B of Gompertz model) of BG and SBP were the highest when CONT was used (300 and 288 ml for BG and SBP, respectively), while for WB the parameter B was the highest when POWD was used (171 ml). However, the differences between inoculums within substrates were not always statistically significant ($P>0.05$). Higher total gas production parameters of substrates incubated in CONT were expected, as it is well known from rumen incubation studies (Roth, 2003; Sivka and Lavrenčič, 2007) that the supplementation of substrates with CWE decreased the gas production. The values of parameter B can be accurately estimated only when prolonged incubation times are used. We suggest that because of such long incubation times the caecum microorganisms had enough time to destroy the coating around CWE (COAT), releasing it into the medium and allowing it to react with (remaining) substrate, microbial enzymes and cell walls. When substrates were incubated in CONT or COAT they had higher specific rates of fermentation (parameter C) then when substrates were incubated in POWD (Table 1), although differences were not always statistically significant ($P>0.05$). The parameters A of BG and WB were always higher when CONT was used, except for SBP where significantly higher ($P<0.05$) parameter A was obtained with the use of POWD (Table 1).

Table 1: Parameters of the Gompertz model of barley grain, sugar beet pulp and wheat bran

Substrate	Inoculum	B† (ml/g DM)	C†	A†
Barley grain (BG)	CONT	300^{a}	11.83^{a}	0.191^{a}
	POWD	278^{abc}	7.45^{b}	0.159^{b}
	COAT	270^{bc}	10.98^{a}	0.186^{a}
Sugar beet pulp (SBP)	CONT	288^{ab}	3.25^{d}	0.128^{c}
	POWD	264^{c}	3.13^{d}	0.155^{b}
	COAT	261^{c}	3.21^{d}	0.124^{c}
Wheat bran (WB)	CONT	163^{d}	4.57^{c}	0.161^{b}
	POWD	171^{d}	3.29^{d}	0.141^{bc}
	COAT	167^{d}	3.59^{d}	0.132^{c}
RMSE§		12.6	0.503	0.0127
Statistical significance				
Substrate		***	***	***
Inoculum		*	***	
Substrate × inoculum			***	**

† B = asymptotic amount of the produced gas (total potential gas production), C = specific gas production rate, A = the decay in specific gas production rate; ^{a,b,c,d} = means in columns with different superscripts are significantly different at the level $P<0.05$; § = root mean square error

Apart from the estimated parameters of gas production (parameters B, C and A) additionally parameters such as the delay of fermentation at the start of incubation (LAG), maximum fermentation rate (MFR), time of maximum fermentation rate (TMFR) and gas produced until 8 h of incubation (Gas8) helped to describe the fermentation pattern of the substrates (Table 2). All these parameters

were significantly affected by the substrate and type of inoculum (P<0.01). However, the only significant interaction (P<0.001) between substrate and type of inoculum was calculated for MFR.

Table 2: Lag phase (LAG), maximum fermentation rate (MFR), time of maximum fermentation rate (TMFR) and total gas production till 8 hours of incubation (Gas8)

Substrate	Inoculum	LAG (h)	MFR (ml/h)	TMFR (h)	Gas8 (ml/g DM)
Barley grain (BG)	CONT	7.7^{a}	21.1^{a}	12.9^{a}	23^{f}
	POWD	6.3^{b}	16.3^{c}	12.6^{a}	35^{e}
	COAT	7.4^{a}	18.5^{b}	12.9^{a}	23^{f}
Sugar beet pulp (SBP)	CONT	1.3^{de}	13.3^{d}	9.5^{b}	88^{b}
	POWD	0.9^{e}	15.1^{c}	7.4^{c}	106^{a}
	COAT	1.3^{de}	11.8^{e}	9.4^{b}	78^{b}
Wheat bran (WB)	CONT	3.2^{c}	9.7^{f}	9.5^{b}	46^{d}
	POWD	1.3^{de}	8.7^{fg}	8.4^{bc}	60^{c}
	COAT	2.1^{d}	8.1^{g}	9.7^{b}	48^{d}
RMSE[§]			0.75	0.68	6.4
Statistical significance					
Substrate		***	***	***	***
Inoculum		***	***	**	***
Substrate × inoculum			***		

^{a,b,c,d} = means in columns with different superscripts are significantly different at the level P<0.05; [§] = root mean square error

Generally, BG had the longest TMFRs (12.6, 12.9 and 12.9 h for POWD, CONT and COAT, respectively), followed by WB (8.4, 9.5, 9.7 and 8.4 h for POWD, CONT and COAT, respectively) and SBP (7.4, 9.5 and 9.4 h for POWD, CONT and COAT, respectively). When the TMFRs within each substrate and between different inoculums were compared, the shortest TMFRs were observed when substrates were incubated in POWD, but the differences were significant (P<0.05) only for SBP (Table 2). Roth (2003) and Sivka and Lavrenčič (2007) incubated different substrates *in vitro* in the rumen fluid containing increasing amounts of CWE powder and noted that the LAGs and TMFRs prolonged and MFRs decreased. Our results are not in accordance with these observations, as LAGs and TMFRs of COAT and CONT were very similar (P>0.05) and those of POWD were always shorter than those of CONT or COAT, especially when WB and SBP were used as substrates. We suppose that these differences could result from: i) higher concentration of CWE used in the studies of Roth (2003) and Sivka and Lavrenčič (2007), ii) substrates used by Roth (2003; soybean meal, rapeseed meal and peas) and Sivka and Lavrenčič (2007; pure cellulose) and iii) from the fact that CWE in the present study was subjected to digestive processes in the stomach and small intestine. Its chemical composition and activity could be changed before it reaches the caecum, from which the inoculum was prepared. On the contrary, in the studies of Roth (2003) and Sivka and Lavrenčič (2007) CWE was not subjected to digestive processes as it was added directly to the inoculum prepared from rumen fluid. Shorter LAGs and TMFRs obtained by incubation of substrates in inocula prepared from rabbit caecum contents suggest that POWD increases the activity of caecal microorganisms. However, the higher microbial activity was not reflected also in the MFRs, where only SBP fermented faster in POWD (15.1 ml/h) than in CONT and COAT (13.3 and 11.8 ml/h, respectively). In the first 8 h of incubation, that correspond to the normal retention time of substrates in the caecum (Gidenne *et al.*, 2000), significantly (P<0.05) greater amounts of gas (Gas8) were obtained with the incubation of all substrates in POWD than in CONT or COAT (Table 2). These results suggest that CWE added to the rabbit diets as powder increase microbial activity in the caecum. Makkar (2003) reported that tannins increased the microbial protein synthesis in the rumen and thus also the rumen microbial activity. We suppose that this could take place also in the rabbit caecum. Thus, next to LAG and TMFR also the Gas8 could serve as a measure of microbial activity in the caecum. Furthermore, CWE in POWD seems to have greater positive effect on pectinolytic microbes and/or enzymes (SBP) than on amylolytic (BG) and hemicellulolytic (WB) microbes (enzymes) as the difference in Gas8s between POWD and CONT were greater for SBP (106 and 88 ml, respectively) than for BG (35 and 23 ml, respectively) and WB (60 and 48 ml, respectively). Blümmel *et al.* (1997) and Getachew *et al.* (1998) reported that the amount of gas produced at the incubation of substrates in rumen fluid is directly related to the amount of short chain fatty acid (SCFA) production. From analogy, the greater Gas8 suggests that the supplementation of rabbit diets with CWE powder increases the SCFA production,

which decreases the caecal pH and in this way have positive effect on the health status of the growing animal (Gidenne, 1997). The LAGs, TMFRs and Gas8 of substrates incubated in COAT did not differ greatly from those incubated in CONT. This suggests that during the coating process the CWE was protected in a manner that does not allow its release at the beginning (in first hours) of incubation in the rabbit caecum. After prolonged incubation (approx. after 20 h) the CWE began to loosen and then it started to affect the fermentation of substrate in a similar manner as CWE added to the diet in powder form (see parameter B in Table 1).

CONCLUSIONS

The main purpose of coating the chestnut wood extract (CWE) was to achieve the protection of tannins against the changes due to the action of hydrochloric acid in stomach and digestive enzymes in the small intestine. This should allow the CWE to provide its beneficial effects (e.g. control of potentially harmful microorganisms) in the large intestine and caecum. However we did not observe any differences at the beginning of fermentation between inocula prepared from caecum contents of animals receiving diets without CWE and those receiving coated CWE, which suggests that the method used for coating CWE was not appropriate. On the contrary, the supplementation of rabbit diets with CWE in form of powder positively affected the gas production until 8 h of fermentation and shortened the lag phase and time of maximum fermentation rate, suggesting that CWE supplemented to the diet as powder reached the caecum and increased the activity of caecal microbes.

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