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IN VITRO FERMENTATION OF DIFFERENT COMMERCIALLY AVAILABLE PECTINS USING INOCULUM FROM RABBIT CAECUM

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Abstract: *In vitro* gas production kinetics of eight different commercially available pectin isolates from apple (4), citrus (2) and sugar beet (2), with a degree of methyl esterification (DMet) ranging from 9 to 73%, were determined twice using the mixture of caecum contents of two rabbits of 78 d of age per repetition as inoculum. The cumulated gas production over 60 h of incubation was modelled with the Gompertz model and the estimated and calculated kinetic parameters of each substrate compared. Total potential gas production (B), gas production till 10 h of incubation, maximum fermentation rate (MFR) and time of maximum fermentation rate (TMFR) were affected (P<0.001) by the DMet, but not by the pectin source. Increasing the DMet of pectins increased total potential gas production. The highest B value was determined for Citrus pectin with 72% DMet (Citrus72, 376 mL/g dry matter (DM)), while the lowest for Citrus pectin with only 10% DMet (Citrus10, 289 mL/g DM). Similar trends were also established for the amount of gas produced up to 10 h of incubation, as Citrus72 produced the highest volume of gas (261 mL/g DM) and Citrus10 the lowest (103 mL/g DM). Increasing DMet increased MFR from 9.9 mL/h for Citrus10 to 34.6 mL/h for Citrus72, while TMFR was shortened with the increasing DMet of pectins from 10.3 h for Citrus10 to 6.0 h for both Citrus72 and 60% DMet Apple pectin.

Key Words: *in vitro* gas production, pectins, degree of methyl esterification, rabbit.

INTRODUCTION

Pectic substances have been determined in citrus, apple and sugar beet pulp in relatively high amounts and the majority of commercially available pectins are isolated from these three sources. They are rich in galacturonic acid, which forms the backbone of two most abundant types of pectins that are thought to be found in all plant species: homogalacturonan (HGA) and rhamnogalacturonan I (RG-I) (Willats *et al*., 2001). The HGA is often methyl esterified. When more than 50% of carboxylic groups of HGA are esterified, pectins are usually defined as highly methyl esterified (HM), otherwise they are defined as modestly or low methyl esterified (LM) pectins (Willats *et al*., 2001). In the second type of pectin, RG-I, rhamnose residues are often substituted with side chains containing neutral residues of arabinose, xylose and/or galactose residues forming arabinans, galactans and arabinogalactans (Willats *et al*., 2001).

Chemical and physical characteristics of pectins depend on the source and methyl ester contents of pectins (Novosel'skaya *et al*., 2000; Gulfi *et al*., 2005) as they determine the stability of gels, water holding capacity and solubility of pectins. Feed with higher contents of pectic substances increased viscosity in small intestine (Volek *et al*., 2005), improved gut barrier function and ileal digestibility of starch and

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reduced caecal frequency of detection of *Clostridium perfringens* (Gómez-Conde *et al*., 2007), increased caecal biomass (Jehl and Gidenne, 1996), short chain fatty acids (Jehl and Gidenne, 1996; García *et al*., 2000; Marounek *et al*., 2007) and *in vitro* gas production (Marounek *et al*., 1997; Lavrenčič, 2007) and decreased mortality in rabbits (Gidenne, 2003; Gómez-Conde *et al*., 2009).

These effects could be partially explained by high pectin fermentability. The faecal digestibilities of uronic acids (eg. galacturonic acid), which are the main constituents of pectins, vary from 30 to 75% depending on fibre source (Gidenne, 1992; García *et al*., 1999). Carabaño *et al*. (2001) found that in rabbits a valuable amount of fibre (up to 40% of NSP) is fermented before the caecum and this is well correlated with fibrolytic activity in intestine and stomach (Marounek *et al*., 1995). Our objective was to compare the *in vitro* fermentation characteristics of three commercially available sources of pectins (citrus, apple and sugar beet pectin) varying in their DMet, using rabbit caecum contents as inoculum.

The *in vitro* procedure of gas production selected is often used in the studies of ruminal fermentation (Menke and Steingass, 1988; Getachew *et al*., 1998) and was modified for pigs (e.g. Bauer *et al*., 2004) and rabbits (Calabro *et al*., 1999; Lavrenčič, 2007), predicting accurately gross energy of rabbit diets (Calabro *et al*., 1999).

MATERIAL AND METHODS

Substrates and in vitro fermentation

Eight different commercially available pectin isolates, deriving from three plant species (apple, citrus and sugar beet) were chosen. These pectins varied in their degrees of methyl esterification (DMet) from 9 to 73%. Commercial name, manufacturer, DMet and source (abbreviation) are reported in Table 1. DMet values were given by manufacturers or calculated as an average from their reported ranges.

Manipulations and selection of animals and the preparation of inoculum were performed according to the methods described by Calabro *et al*. (1999) and Lavrenčič (2007). In each of the two repetitions

Table 1: Commercial name, manufacturer, source, degree of methyl esterification (DMet) and abbreviation of pectins used in the experiment.

Commercial name	Manufacturer	Source	DMet (%)	Abbreviation
Art. No. P-8471	Sigma Aldrich (Germany)	Apple	9	Apple9
CLASSIC AU 701	Herbstreith & Fox (Neuenbürg, Werder, Germany)	Apple	40	Apple40
Green Ribbon 150° US-SAG	Obipectin (Bishofszell, Switzerland)	Apple	60	Apple60
CLASSIC AU 201 US	Herbstreith & Fox (Neuenbürg, Werder, Germany)	Apple	73	Apple73
Genu® pectin type BETA	CPKelco (Atlanta, United States)	Beet	55	Beet55
BETAPEC RU 301	Herbstreith & Fox (Neuenbürg, Werder, Germany)	Beet	57	Beet57
Genu® pectin type LM-5 CS	CPKelco (Atlanta, United States)	Citrus	10	Citrus10
Citrus Genu® pectin type 150 USA-SAG	CPKelco (Atlanta, United States)	Citrus	72	Citrus72

Abbreviation consists of the source (eg. apple) and DMet (eg. 60 for 60%).

freshly collected samples of the caecum content were used to prepare the inoculum. Two 78 d old New Zealand White rabbits (Slovenian meat line SIKA) showing normal weight gain were randomly chosen in each repetition prior to slaughtering and were fed the commercial compound feed (Krka, Novo mesto, Slovenia) containing (on dry matter basis (DM); g/kg): crude protein 201, crude fat 22, crude fibre 155, ash 98, neutral detergent fibre 334, acid detergent fibre 190 and acid detergent lignin 51. The diet was offered *ad libitum* from weaning at 35 d of age. The feed was removed at 18:00 h on the day before slaughtering, but water was still available *ad libitum* (Calabro *et al*., 1999). The animals were sacrificed between 8:00 and 8:30 h and the caeca were isolated by tying off the two extremities with nylon string to prevent movement of the digesta.

Caeca were transported to the laboratory within 10 min in a prewarmed (39 °C) container. The caecum contents from both sacrificed animals were then mixed together in a 200 mL beaker. Depending on the number of syringes, the exact quantity of caecum contents was weighed into another 200 mL beaker, placed on a balance and diluted with 150 mL of a buffer solution prepared according to Menke and Steingass (1988). The suspension was then squeezed through four layers of cotton gauze into a 2 l flask with the remaining buffer. The dilution of caecum contents in the buffer solution was equal to 1:50 (w/v; 0.02 g of caecum contents/mL of buffer solution). All manipulations (mixing, weighing, diluting, filtering through gauze and constitution of inoculum) and the filling of syringes were done under the constant flushing of the oxygen free CO_2 gas to assure anaerobical conditions and the temperature was always kept at 39.0 °C. All syringes were filled no later than 40 min after slaughter.

In vitro gas production was determined according to the procedure described by Menke and Steingass (1988). Approximately 175 mg of air dry pectin isolates were anaerobically incubated at 39 °C in triplicate in a 100 mL glass syringe containing 30 mL of inoculum. The production of gas resulting from microbial fermentation was measured manually by reading the position of the bottom end (marked with line) of the piston after 0, 2, 4, 6, 8, 10, 12, 24, 36, 48 and 60 h. In each repetition two syringes with blank samples (syringes without substrate) were also incubated.

Calculations and statistical analysis

The values of gas produced at different times of incubation from inocula prepared from the caecum contents of both repetitions were corrected for the amount of gas produced from blank samples at the corresponding times within each repetition and type of inoculum. These values were also corrected for the DM contents of pectin isolates. The values corrected in such a manner were then fitted to the Gompertz model (Bidlack and Buxton, 1992; Lavrenčič *et al*., 1997):

$$Y_t = B \times e^{-C \times e^{-At}}$$

where Y_t was the gas produced (mL/g DM) at time t, B was the asymptotic amount of the produced gas (maximum amount of produced gas or total potential gas production; mL/g DM), C is the specific gas production rate as affected by t, and is governed by a constant A describing the decay in specific gas production rate (caused by diminishing growth rate of microorganisms and increasing substrate limitation as reflected in gas production; Beuvink and Kogut, 1993), and »t« is time in hours. Parameter values and curve fitting were estimated by the Marquard compromise of a non-linear regression method, using SAS software (Proc NLIN) (SAS, 1994).

The cumulative amount of gas produced within 10 h of fermentation (Gas10; mL/g DM) was calculated by inserting the time “t” of 10 h into the above equation. The variation in gas production rates was obtained by calculating the first derivative of the Gompertz model with respect to the time of incubation.

The time of maximum fermentation rate (TMFR; h) and the maximum fermentation rate (MFR; mL/h) were calculated using equations reported by Lavrenčič (2007).

Data concerning fermentation kinetic parameters (parameters B, C, A, Gas10, MFR and TMFR) were tested for significance by covariance analysis using the Scheffe test to compare the pectin sources.

$$Y_{ij} = \mu + S_i + b \times DMet + e_{ij}$$

where Y_{ij} was the value, μ the mean, S_i the effect of source of pectin isolate (apple, beet or citrus), b the regression coefficient of the DMet and e_{ij} the residual errors. The interaction between pectin source and DMet was not significant and was omitted from the statistical model. All statistical analyses were performed using the GLM and REG procedures of the SAS (SAS, 1994).

RESULTS

Total potential gas production (B) of pectins increased (P<0.001) by 0.88±0.17 mL/g DM per each unit that DMet increased (Table 2 and Figure 1). The largest amount of gas was produced by Citrus72 pectin, while the lowest by Citrus10 pectin. Source of pectin did not influence the B value, which varied between 338 and 350 mL/g DM for Citrus and Apple pectins, respectively. DMet did not influence the specific gas production rate (C). Sugar beet pectins had higher (P<0.05) C values (5.61) than apple (4.31) and citrus pectins (3.60). The decay in the specific gas production rate (A) increased with increasing

Table 2: Estimated gas production parameters of different commercially available pectic substances incubated *in vitro* in the inocula prepared from the rabbit caecum contents (n=2).

		Fermentation kinetic parameters[1]		
Substrate	DMet[2] (%)	B (mL/g DM)	C	A
Apple9	9	337	5.41	0.197
Apple40	40	333	3.59	0.182
Apple60	60	356	4.38	0.245
Apple73	73	369	3.86	0.196
Beet55	55	339	5.45	0.261
Beet57	57	341	5.76	0.260
Citrus10	10	289	2.62	0.093
Citrus72	72	376	4.54	0.250
RSD[3]		19.3	0.897	0.0186
Statistical significance				
Source[4]		0.17	0.004	0.011
Apple *vs*. Sugar beet		–	0.038	0.084
Apple *vs*. Citrus		–	0.300	0.313
Citrus *vs*. Sugar beet		–	0.005	0.011
DMet[2]		<0.001	0.720	<0.001

[1] B: total potential gas production, C: specific gas production rate as affected by t and governed by the constant A, and A: the decay in specific gas production rate, [2]DMet: degree of methyl esterification,[3]RSD: residual standard deviation, [4]source of pectin (apple, sugar beet or citrus). Interactions between source and DMet were not significant.

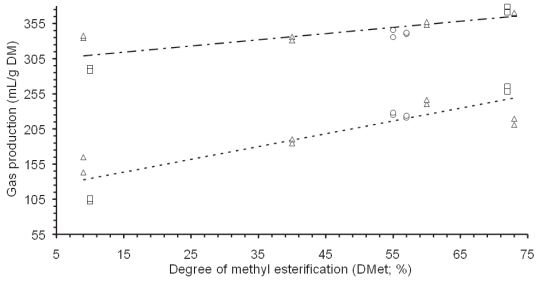

Figure 1: Relationship between degree of methyl esterification (DMet; %) and total potential gas production (B - -; y=301.12(±9.13)+0.88(±0.17) ×DMet; R^2=0.52) and gas produced till 10 h of incubation (Gas10 - - -; y=116.23(±11.72)+1.82 (± 0.22)×DMet; R^2=0.74). n=16 (△=apple pectin; ○= sugar beet pectin; □= citrus pectin).

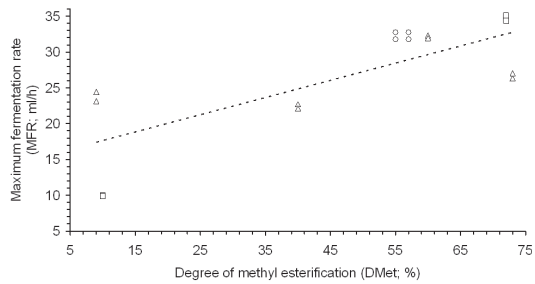

Figure 2: Relationship between degree of methyl esterification (DMet; %) and maximum fermentation rate (MFR; y=15.25(±2.38)+0.24(±0.05)×DMet; R^2=0.56). n=16 (△=apple pectin; ○= sugar beet pectin; □= citrus pectin).

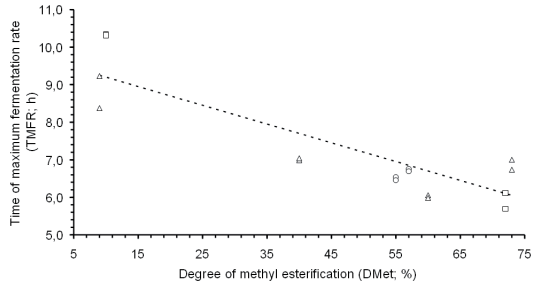

Figure 3: Relationship between degree of methyl esterification (DMet; %) and time of maximum fermentation rate (TMFR; y=9.703(±0.375)-0.050 (±0.007)×DMet; R^2=0.70)). n=16 (△=apple pectin; ○= sugar beet pectin; □= citrus pectin).

DMet (P<0.001). Sugar beet pectins had higher (P=0.011) A value (0.249) compared to Citrus pectins (0.172), while both had similar A values compared to Apple pectins (P>0.05). The highest A value was determined for Beet55 while the lowest for Citrus10 pectin (Table 2).

Parameters calculated from B, C and A (Gas10, MRF and TMFR), were influenced by DMet (P<0.001, Figures 1, 2 and 3) but not affected by source of pectin (P>0.05). With the increased DMet of pectins Gas10 and MFR increased (P<0.001) by 1.82±0.22 mL/g DM and 0.24±0.05 mL/h, respectively, while TMFR values decreased by 0.050±0.007 h (P<0.001).

DISCUSSION

Previous *in vitro* gas production studies working with pig and human faeces as inoculums and with pectins differing in their DMet demonstrated that pectins with higher DMet contents produced greater amounts of gas than those with lower DMet contents (Drochner *et al*., 2004; Gulfi *et al*., 2005). This is in agreement with the results obtained in the present study. Siragusa *et al*. (1988) and Drochner *et al*. (2004) stated that the greater amounts of gas produced *in vitro* from high DMet pectins come from methoxyl groups linked to galacturonic acid, which are converted into methanol and then rapidly metabolised into gaseous methane, thus contributing to the amount of gas produced during incubation.

However, Nyman and Asp (1982) suggested that rats ferment LM pectin (DMet 37%) more efficiently than HM pectin (DMet 74%), as the recoveries of uronic acids in faeces were 19% and 25% for LM and HM pectins, respectively. Similarly, Dongowski and Lorenz (1998) and Dongowski *et al*. (2002) incubated LM (DMet 34.5 and 34.4%, respectively) and HM citrus pectins (DMet 70.8 and 66.0%, respectively)

with inoculum prepared from human and rat faeces, and established that LM pectins were degraded faster and produced more short chain fatty acids than HM pectins. Furthermore, Olano-Martin *et al*. (2002) determined the higher growth rates of pure bacterial cultures, isolated from human faeces, when they were incubated on LM pectins (DMet 8%) than on HM pectins (DMet 66%) and thus higher degradation of LM pectins. According to these authors, the higher disappearance rates of LM pectins and higher growth of faecal bacteria would be related to the structure of LM pectins, in which GalA is more directly exposed to microorganisms and their enzymes and is thus rapidly fermented, while in HM pectins many methoxyl groups have to be removed before the galacturonic acid backbone is fermented.

The differences between results could be attributed to i) *in vitro* conditions of the different experiments (e.g. the composition of the fermentation buffer and the ratio between substrate, buffer and microflora in the inocula), ii) the microbial composition of the inocula which is strongly affected by the diet of the human or animal donors, iii) the method used (eg. gas production *vs*. substrate disappearance) (Dongowski and Lorenz, 1998; Dongowski *et al*., 2002; Gulfi *et al*., 2005 and 2006). Blümmel *et al*. (1997) stated that the use of single method does not provide complete information on the *in vitro* fermentation and suggested that a combination of measurements should be used.

The DMet content of pectins also affected TMFR. All commercially available pectins used in our study had TMFR shorter than or near to 10 h (from 6.0 to 10.3 h), which is the retention time of substrates in the caecum of rabbits (Gidenne *et al*., 2000). We consider that TMFRs in *in vivo* conditions are much shorter because the substrates are hydrated and colonized with microbes before they reach caecum, while in *in vitro* conditions the hydration and microbial colonization starts only when the incubation starts. Furthermore, longer *in vitro* than *in vivo* TMFR are expected because of the dilution of the inocula in the buffer (Bindelle *et al*., 2007), decreased concentrations of active bacteria in the inoculum and because of the lower amounts of nutrients in the inoculum to which microorganisms are adapted (Campbell *et al*., 2002; Bindelle *et al*., 2007).

According to Beuvink and Kogut (1993) the C and A parameters produced by the Gompertz model describe the specific fermentation rate and the constant factor of microbial efficiency, but the biological meaning of these parameters is not clear and we can not give definite conclusions on the effect of the pectin source on them.

In conclusion, the degree of methyl esterification (DMet) of pectins showed a much greater influence on *in vitro* gas production traits than the pectin source. However, further studies would be necessary to clarify the contradictory results observed in the literature.

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