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THE EFFECT OF SWEET CHESTNUT EXTRACT (FARMATAN®) ON KINETICS OF IN VITRO CELLULOSE FERMENTATION

UTJECAJ EKSTRAKTA KESTENA (FARMATAN®) NA KINETIKU IN VITRO FERMENTACIJE CELULOZE

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ABSTRACT

Four different concentrations [0 (control), 0.33, 0.67 and 1.33 mg/ml medium)] of sweet chestnut extract (Farmatan®, Tanin, Slovenia) were used to investigate their effect on the kinetics of *in vitro* fermentation of pure cellulose (BWW 40, J. Rettenmaier & Söhne, Germany). Gas produced was measured at 0, 2, 4, 6, 8, 10, 12, 24, 36, 48, 72 and 96 h after the start of incubation. Gompertz model was used to estimate kinetic parameters "B" (total potential gas production), "C" (relative degradation rate) and "A" (constant decay in relative degradation rate). First and second derivative of Gompertz model were used to calculate the maximum fermentation rate (MFR) and time of maximum fermentation rates (TMFR), respectively. Comparing with the control (550.8 ml/g DM) the total potential gas production significantly decreased ($p < 0.05$) when 0.33 and 0.67 mg of Farmatan® were added to the medium (474.0 ml/DM and 504.2 ml/g DM, respectively). On contrary, the highest concentration of Farmatan® (1.33 mg/ml medium) did not decrease the total potential gas production. Maximum fermentation rate of cellulose (21.4 ml/h) occurred after 13 h of incubation. Increasing concentrations of Farmatan® decreased MFR ($p < 0.05$) from 21.4 ml/h (control) to 16.6 ml/h (0.33 mg/ml medium), 18.4 ml/h (0.67 mg/ml medium) and 10.5 ml/h (1.33 mg/ml medium). Farmatan® significantly increased ($p < 0.05$) TMFR. The increase was the greatest when 0.67 mg of Farmatan® was added to the medium (29.2 h). Lag phase of cellulose (4.4 h in control) did not change significantly ($p < 0.05$) with increasing concentrations of Farmatan®.

Keywords: sweet chestnut extract, tannins, fermentation, cellulose

INTRODUCTION

Tannins are phenolic compounds commonly found in forage legumes, shrubs and trees, where they act as protecting agents against the attacks of herbivores and pathogenic microorganisms. Their main characteristic is the formation of complexes with proteins, amino acids, polysaccharides, metal ions, vitamins, bacterial cell membranes and enzymes involved in protein and carbohydrate

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digestion (Mole and Waterman, 1987., Butter et al., 1999., Makkar, 2003). The formation of complexes between tannins and above mentioned substrates has both, beneficial and adverse effects on herbivorous animals (Kumar and Vaithiyanathan, 1990., Butter et. al., 1999., Makkar, 2003). Low concentrations of tannins (less than 5 g/kg of ration dry matter) increase microbial protein synthesis and decrease protein degradability of dietary protein in the rumen, thereby reducing methane production and nitrogen excretion to the environment. Ruminants are thus better supplied with essential amino acids which enhance milk, meat and wool production (Makkar, 2003).

The effect of tannins on rumen microbial fermentation of carbohydrates was not extensively studied. Reed (1995) and McSweeney et al. (2001) reported that tannins form hydrogen bonds with cellulose, hemicelluloses, starch and pectin. The strength of binding of tannins is influenced by solubility, molecular size and conformational flexibility of carbohydrates (Haslam, 1989). Tannins reduce fibre digestion by inhibition of bacterial enzymes involved in the carbohydrate metabolism (cellulases, amylases and galactosidases) or by substrate deprivation (binding with lignocellulose, cellulose, hemicelluloses, starch and pectin's) or both (Butter et al., 1999., McSweeney et al., 2001). However, despite the antimicrobial properties of tannins, many microorganisms can grow and develop on tannin-rich materials (Scalbert, 1991).

In high concentrations tannins can exert toxic effects, especially when hydrolysable tannins are consumed. Rumen microbes are capable of degrading hydrolysable tannins (Makkar, 2003) and their degraded products are absorbed from the intestines. Small amounts of these products are efficiently detoxified in the liver, while higher amounts can cause poisoning of the animal (Makkar, 2003).

Fermentative activity of rumen microorganisms could be estimated with several different methods such as microbial count (Williams et al., 2001), production rates of microbial protein synthesis (ATP production rate; Venkateswaran et al., 2003) and/or fermentation end products (volatile fatty acids; Blummel et al., 1997). The whole microbial population activity could be measured also with the measurements of accumulating gas at different incubation times (Menke and Steingass, 1988., Williams et al., 2001). The technique is carried out under strictly anaerobic conditions and is being used to examine the effects of different substrates and feed additives on activity of rumen microflora (Menke and Steingass, 1988., Lavrenčič and Stefanon, 2001., Getachew et al., 2004). Changed rumen microbial activities could be determined by estimating parameters of *in vitro* fermentation kinetics, such as extent of gas production and fermentation rates and by calculating additional parameters such as maximum fermentation rate and time of maximum fermentation rate.

The objective of this study was to investigate the effect of different concentrations of sweet chestnut extract (Farmatan®) on rate and extent of *in vitro* fermentation of pure cellulose. In addition, we want to determine also the effect of above mentioned concentrations of chestnut tannins on maximum fermentation rate and time of maximum fermentation rate.

MATERIAL AND METHODS

Cellulose (BWW40; J. Rettenmaier and Söhne, Germany), alone and with different concentrations (0, 0.33, 0.67 and 1.33 mg/ml medium) of sweet chestnut wood extract (Farmatan®; Tanin Sevnica, Slovenia) was incubated in triplicate in 100 ml glass syringes according to the method of Menke and Steingass (1988). Glass syringes containing substrates were filled with 30 ml buffered rumen liquor (10 ml rumen liquor and 20 ml buffer) and incubated at 39°C in water bath. Three empty syringes (blank samples) and three syringes containing ryegrass hay (standard sample) were also incubated and used after incubation for gas production corrections. Rumen liquor was collected from two rumen-fistulated castrated adult Jezersko-solčavska male sheep. Animals were fed with a diet containing hay (*ad libitum*), concentrate (400 g/d; 18 % CP) and mineral-vitamin mix (20 g/d) to cover their daily nutrient needs according to DLG (1997). Gas measurements were taken at two hours interval for the first 12 hours then each 12 hours up to 48 hours and each 24 hours up to 96 hours.

Gas production data were fitted to the Gompertz model (Bidlack and Buxton, 1992; Lavrenčič et al., 1997):

$$y\,(t) = B\,e^{-C\,e^{-A\,t}} \qquad (1)$$

where *y(t)* is the cumulative gas production (ml) at time *t*, *B* is total potential gas production, *C* is relative fermentation rate, affected by a constant *A* describing the decay in relative fermentation rate and *t* is time in hours. Parameter estimates of *B*, *C* and *A* were obtained with the Marquardt compromise of a non-linear regression method using the SAS software (PROC NLIN; SAS, 2001). The variation in gas production rates was obtained by calculating the first derivative of the Gompertz model with respect to the time of incubation:

$$\frac{dY}{dt} = B \times C \times A \times e^{-At} \times e^{-C \times e^{-At}} . \qquad (2)$$

The time of maximum fermentation rate (TMFR; h) was calculated by setting the second derivative of the Gompertz model equal to 0 and solving for »t«:

$$\frac{d^2Y}{dt^2} = A \times B^2 \times C^2 \times (e^{-At})^2 \times e^{-C \times e^{-At}} -$$

$$- A \times B \times C^2 \times e^{-C \times e^{-At}} = 0 . \qquad (3)$$

The maximum fermentation rate (MFR; ml/h) was then calculated by inserting the corresponding TMFR value into the first derivative equation.

The delay in fermentation at the start of incubation (LAG; hours) was obtained from the equation:

$$LAG = \frac{\log(C) - 1}{A} . \qquad (4)$$

Data concerning fermentation kinetic parameters (parameters B, C, A, LAG, MFR and TMFR) were tested for significance by analysis of variance using the Scheffe test:

$$Y_{ij} = \mu + C_i + e_{ij} \qquad (5)$$

where Y_{ijk} is the value, μ the mean, C_i the effect of chestnut tannin concentration and e_{ij} the residual errors. All statistical analyses were performed using the of GLM procedure of the SAS (SAS, 2001).

RESULTS AND DISCUSSION

Estimated and calculated parameters of *in vitro* fermentation of cellulose alone and with different concentrations (0, 0.33, 0.67 and 1.33 mg/ ml medium) of sweet chestnut wood extract (Farmatan®) are presented in Table 1. When 0.33 and 0.67 mg of Farmatan® were added to the mixture of buffer and rumen liquor the total potential gas production decreased from 531.4 ml/g DM (control) to 474.0 ml/g DM and 504.2 ml/g DM, respectively. The addition of highest concentration of Farmatan® (1.33 mg/ml medium) did not decrease the total potential gas production. Our observations are slightly different of those reported by Roth (2003), where increased concentrations of Farmatan® decreased gas production of soybean meal. On contrary, Lavrenčič (2001) reported no significant differences in total gas productions of soybean meal incubated *in vitro* with rising concentrations of Farmatan®. Silanikove et al. (2001) and Reed (1995) reported that the binding strength in tannin-protein and tannin-carbohydrate complexes depends on the characteristics of tannins, proteins and carbohydrates, e.g. molecular weight, tertiary structure, isoelectric point and compatibility of binding sites. These could be the reasons why our observations differed from published ones.

Addition of Farmatan® (Table 1) increased relative degradation rate of cellulose from 4.7 (control) to 18.4 (0.67 mg/ml medium). Similarly to total potential gas production, the addition of highest concentration of Farmatan® (1.33 mg/ ml of medium) did not cause significant changes in relative degradation rate. On contrary, rising concentration of Farmatan® significantly decreased constant decay in relative degradation rate, which was reduced for almost 50 % when the highest concentration of Farmatan® was used.

Table 1. Estimated and calculated kinetic parameters of *in vitro* fermentation of cellulose with increasing concentrations of sweet chestnut wood extract (Farmatan®)

Tablica 1. Procijenjeni i izračunati kinetički parametari in vitro fermentacije celuloze sa povećavanjem koncentracije ekstrakta kestena (Farmatan®)

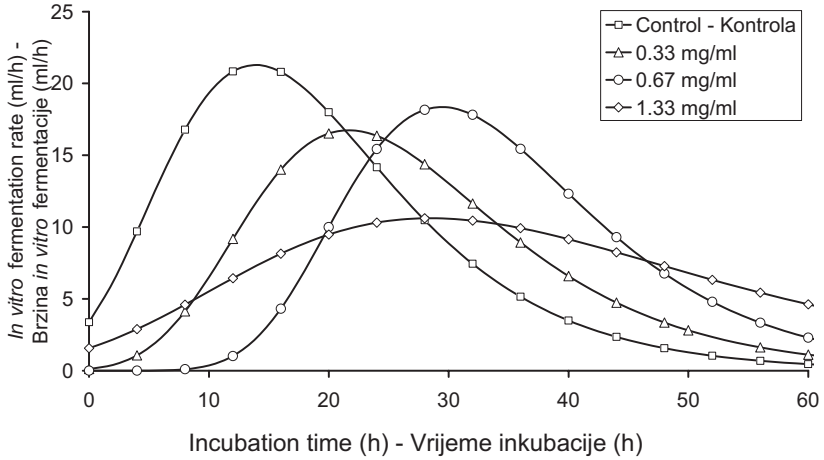
Farmatan concentration Koncentracija Farmatana (mg/ml)	Estimated kinetic parameters Procijenjeni kinetički parametari			Calculated kinetic parameters Izračunati kinetički parametari		
	B† (ml/g DM) B (ml/g ST)	C†	A†	LAG§ (h)	TMFR§ (h)	MFR§ (ml/h)
0 (control - kontrola)	531,4[a]	4,7[a]	0,116[a]	4,7[a]	13,3[a]	22,6[a]
0.33 mg/ml	474,0[b]	8,0[a]	0,096[b]	11,3[b]	21,8[b]	16,7[b]
0.67 mg/ml	504,2[c]	18,4[b]	0,100[b]	19,1[c]	29,2[c]	18,4[b]
1.33 mg/ml	537,3[a]	4,6[a]	0,055[c]	9,7[b]	28,0[c]	10,8[c]

[abc] means with different letters in the same column are significantly different ($p < 0.05$)

† B = maximum amount of produced gas - maksimalna količina stvorenog plina; C = specific gas production rate as affected by t (incubation time), and is governed by a constant C - specifična brzina stvaranja plina na koju utiče t (čas inkubacije), te ga usmjerava konstantni faktor A; A describes the decay in specific gas production rate - A opisuje smanjenje specifične brzine fermentacije

§ LAG = lag time (h) - vrijeme zaostatka početka fermentacije; TMFR = time of maximum fermentation rate (h) - vrijeme u kojem je brzina fermentacije najveća (h); MFR = maximum fermentation rate (ml/h) - maksimalna brzina fermentacije (ml/h)

Cellulose lag phase was prolonged when Farmatan® was added to medium. Lag phase was the greatest when medium contained 0.67 mg of sweet chestnut wood extract per ml (19.1 h). The most probable cause for prolonged LAG is the formation of bonds between tannins and microbial enzymes and between tannins and substrate. Because of these bonds the substrate is not degraded by rumen bacterial enzymes (Scalbert, 1991., McSweeney et al. 2001). However, if the complexes between tannins and substrates and/or microbial enzymes are formed, than the LAG will be infinite. However, O'Donovan and Brooker (2001) suggested that microorganisms form a protective barrier around their cells on which the tannins are preferentially bound. This protective microbial barrier prevents binding between tannins and microbial enzymes and immediately after the concentration of the tannins in the medium is low enough the fermentation starts.



Graph 1. Cellulose fermentation rate at different Farmatan® concentrations
Graf 1. Brzina fermentacije celuloze kod različitih koncentracija Farmatana®

Increasing amounts of Farmatan® significantly increased time of maximum fermentation rate (TMFR) of cellulose and significantly decreased maximum fermentation rate (MFR) of cellulose. Maximum fermentation rate of pure cellulose (control) occurred after 13 hours of incubation and had the MFR of 22.6 ml per hour. Comparing with control (13.3 h) the TMFR increased to more than 28 hours ($p < 0.05$) when the concentrations of Farmatan® were 0.67 and 1.33 mg/ml medium and MFR decreased to 16.7 ml/h, 18.4 ml/h and 10.8 ml/h when the concentrations of Farmatan® were 0.33, 0.67 and 1.33 mg/ml of medium respectively (Figure 1). This is in accordance with the results of Roth (2003), who observed the decrease in MFR with increasing concentrations of sweet chestnut tannin extract (Farmatan®). Furthermore, Zimmer in Cordesse (1996) also reported decreased *in sacco* degradation rate when sweet chestnut extracts were added in the diet of sheep and goats.

CONCLUSION

Sweet chestnut extract (Farmatan®) added to the inoculum (buffer and rumen liquor) altered *in vitro* fermentation of cellulose. The most prominent changes were noted in the time of maximum fermentation rate (TMFR) which was 13.3 hours when cellulose was incubated in the medium without Farmatan®. With the highest amounts of sweet chestnut wood extracts (0.67 and 1.33 mg/ml of medium) the TMFR prolonged to 29.2 and 28.0 hours, respectively. Supplementation of Farmatan® to the medium decreased maximum fermentation rates (MFR) of cellulose from 22.6 ml/h to 10.8 ml/h when 1.33 mg of Farmatan® was added. We can conclude that Farmatan® modified the kinetic parameters of *in vitro* cellulose fermentation, presumably by forming unfermentable complexes with substrate (cellulose) and by decreasing the activity of microorganisms and/or microbial enzymes.

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SAŽETAK

Četiri različite koncentracije [0 (kontrola), 0.33, 0.67 i 1.33 mg/ml medija)] ekstrakta slatkog kestena (Farmatan®, Tanin, Slovenija) korištene su za istraživanje učinka na kinetiku *in vitro* fermentaciju čiste celuloze (BWW 40, Rettenmaier i Söhne, Germany). Proizvodnja plina mjerena je 0, 2, 4, 6, 8, 10, 12, 24, 36, 48, 72 i 96 sati nakon početka inkubacije. Model „Gomperz" je korišten kako bi se utvrdili parametri kinetike „B" (ukupna potencijalna proizvodnja plina), „C" (relativno vrijeme razgradnje) i „A" (konstantno nestajanje u relativnom vremenu razgradnje). Prva i druga derivacija „Gomperz" modela je korištena kako bi se izračunala maksimalna brzina fermentacije (MBF) i vrijeme maksimalnih brzina fermentacije (VMBF). U usporedbi s kontrolom (550.8 ml/g ST), ukupna potencijalna proizvodnja plina značajno se smanjila ($p < 0.05$) kada je dodano 0.33 i 0.67 mg Farmatana® u medij (473.0 ml/ST i 504.2 ml/ST, za svako posebno). Nasuprot tome, najviša razina Farmatana® (1.33 mg/ml medija) nije smanjila ukupnu potencijalnu proizvodnju plina. Maksimalna brzina fermentacije celuloze (21.4 ml/h) nastupila je 13 sati nakon inkubacije. Povećanje koncentracije Farmatana® smanjilo je MFR ($p < 0.05$) od 21.4 ml/h (kontrola) na 16.6 ml/h (0.33 mg/ml medija), 18.4 ml/h (0.67 mg/ml medija) i 10.5 ml/h (1.33 mg/ml medija). Farmatan® je značajno povećao ($p < 0.05$) TMFR. Povećanje je bilo najviše kada je u medij dodano 0.67 mg Farmatana® (29.2 h). Lag faza celuloze (4.4 h u kontroli) nije se značajno ($p < 0.05$) promijenila s povećanjem koncentracije Farmatana®.

Ključne riječi: ekstrakt slatkog kestena, tanini, fermentacija, celuloza