Effects of Surfactant Tween 80 on Forage Degradability and Microbial Growth on the *In vitro* Rumen Mixed and Pure Cultures

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ABSTRACT: Effect of a surfactant Tween 80 on the bacterial growth in the rumen was examined on the *in vitro* pure cultures of *Streptococcus bovis*, *Selenomonas ruminantium*, *Butyrivibrio fibrisolvens*, *Prevotella ruminicola*, *Megasphaera elsidenni*, *Fibrobacta succinogenes*, *Ruminanococcus albus* and *Ruminococcus flavefaciens*. Dry matter degradability (DMD), concentrations and compositions of volatile fatty acids (VFA), and the most probable number (MPN) of cellulolytic bacteria and total number of bacteria in the presence of Tween 80 were also examined on the *in vitro* rumen mixed culture either with barley grain or orchardgrass hay. The growth of *S. bovis*, *S. ruminantium*, *B. fibrisolvens*, *P. ruminicola*, *M. elsidenni* and *F. succinogenes* were significantly higher ($p<0.05$) at over 0.05% concentrations of Tween 80 than those of the control cultures, while was not changed with *R. albus* and *R. flavefaciens*. With rumen mixed culture the DMD of barley grain and orchardgrass hay was significantly higher ($p<0.05$) at a 0.2% concentration of Tween 80 than the control, being reflected in the significantly higher ($p<0.05$) VFA production (mmol g^{-1} DDM) with orchardgrass hay. The higher ($p<0.05$) ratio of propionate to acetate at a 0.2% concentration of Tween 80 was also observed with orchardgrass hay, showing a similar trend with barley grain. No changes in the total bacterial number and MPN of cellulolytic bacteria were observed. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 5 : 672-676)***

Key Words : *In vitro* Rumen Degradability, Microbial Growth, Surfactant Tween 80

INTRODUCTION

Strategies to improve nutritional value of feedingstuff such as chemical (Goto et al., 1991, 1993, 1998; Goto and Yokoe, 1996; Vadioloo 2000), physical (Hai et al., 1998, 1999) and biological (Yamada et al., 2000a, 2000b) treatments have been extensively studied. And also applied for improving rumen voluntary intake and digestibility of fibrous and low quality roughages. The loosening and/or partial breakdown of rigid cell wall structure of forage resilts in the greater accessibility of fiber-degrading bacteria and their associated enzymes to forage plants. Recently, surfactants have been receiving a wide recognition as a newly proposed method, which can facilitate the enzyme-substrate interactions. For example, surfactants improve enzyme activity (Fendler and Fendler, 1975; Castanon and Wilke, 1981; Kim et al., 1982; Ooshima et al., 1986; Helle et al., 1993; Goto et al., 2002). A surfactant Tween 80 increased DMD of plant fractions of young and matured orchardgrass by a cellulolytic commercial enzyme by 5-35% units, showing the consistency of the improvement of enzymatic degradability with those of their water and enzyme-holding capacities (Goto et al., 2002). It is therefore suggested that adsorption and orientation of the surfactant molecules at the solid-liquid interface could render the substrate readily wettable by the enzymes, thereby providing a highly localized substrate concentration. No study, however, seems to have been made on the effects of surfactants on the rumen digestion with the objective of improving the microbial growth and DMD of forage plant in the rumen by adding various concentrations of surfactants.

The aim of this study is to examine effects of a surfactant Tween 80 on the growth of non-cellulolytic and cellulolytic pure cultures incubated *in vitro* with barley (*Hordeum vulgare* L.) grain and orchardgrass (*Dactylis glomerata* L.). Dry matter degradability (DMD) and production and composition of volatile fatty acids (VFA) of barley grain and orchardgrass hay were also examined on rumen mixed culture with different concentrations of Tween 80.

MATERIALS AND METHODS

The *in vitro* incubation of pure cultures in the rumen

Eight pure cultures of *Streptococcus bovis* (45S1), *Selenomonas ruminantium* (L100), *Butyrivibrio fibrisolvens* (L139), *Prevotella ruminicola* (JB29), *Megasphaera elsidenni* (B159), *Fibrobacta succinogenes* (85), *Ruminonococcus albus* (7) and *Ruminococcus flavefaciens* (Fd1) were examined for the cell growth at three concentrations (v/v; none, 0.05% and 0.1%) of Tween 80. These cultures were obtained from the culture collection of Lethbridge Research Center (Canada), developed and

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Table 1. Chemical composition of barley grain and orchardgrass hay

Substrate	Chemical composition (g kg^{-1}DM)				
	Crude protein	Starch	NDF	ADF	ADL
Barley grain	93.7	620.3	103.6	16.1	1.8
Orchardgrass	145.4	71.8	508.1	230.3	23.4

NDF: neutral detergent fiber. ADF: acid detergent fiber. ADL: acid detergent lignin.

regenerated at 37°C for 1 day (d) in a 20 ml test tube containing deoxidized Dehority artificial media (Dehority, 1965) with ground barley grain or orchardgrass hay.

Cultures of *S. bovis*, *S. ruminantium*, *B .fibrisolvens*, *P. ruminicola* and *M. elsidenni* were anaerobically incubated with a 1% barley grain in the deoxidized artificial medium of 300 ml round bottle flask. *F. succinogenes*, *R .albus*, and *R. flavafaciens* were similarly grown but with a 1% orchardgrass hay. Each of the cultures was sampled in triplicate at 4, 8, 12, 16 and 20 h and used for measurements of the medial pH and microbial growth. At each of the sampling time, the same volume of medium adjusted to the concentration of Tween 80 was replaced in the media pH. The pH of the sample culture was measured immediately using a pH meter. A portion of the sample was first centrifuged at a low speed (3,000 rpm.) to remove feed particles, and the microbial cell pellet was then quantitatively collected by the centrifugation of the supernatant at a high speed (10,000 rpm.). The pellet was washed with distilled water three times by centrifugation and kept at -30°C until appropriate analysis.

The *in vitro* incubation of rumen mixed culture

The *in vitro* incubation of rumen mixed culture with barley grain and orchardgrass hay was examined for the total bacterial number and MPN, DMD, and VFA production and composition at different concentrations (v/v; none, 0.01% and 0.2%) of Tween 80.

Barley grain or orchardgrass hay (ca. 500 mg) was accurately weighed into a 30 ml bottle with butyl rubber stopper and aluminium seal, suspended in deoxidized Dehority medium (excluded carbon source; Dehority, 1965). This was anaerobically incubated at 37°C under the in vitro condition with rumen mixed culture (v/v, 1:9 rumen mixed culture/Dehority buffer solution). Each treatment (3 concentrations×2 substrates) were run at five replications, and the sample was taken at three time points of 2, 8 and 24 h and 8, 24 and 48 h for the barley and orchardgrass, respectively.

Rumen mixed culture was prepared from the rumen fluid of a rumen-fistulated lactating dairy cow fed on diets consisting 60% alfalfa hay and 40% concentrates of DM basis. The rumen content was taken before a morning feeding, strained through five layers of cheesecloth, and anaerobically allowed to stand for 30 min. at 37°C in order to discard large feed particles. The Dehority buffer solution was prepared by deoxidized method using bubbling CO_2 gas and autoclaved at 121°C for 20 min and dispensed into each bottle under CO_2 gas flushing just before the inoculation.

Bacterial growth, DMD, VFA and MPN determinations

Bacterial protein was determined by means of Bio Rad assay using a microplate reader (Bradford, 1976). The DMD was measured by weighing DM residues on glass crucible (GA3) recovered after the *in vitro* incubation. Concentrations of VFA (acetate, propionate, butyrate and valerate) were determined by a gas chromotography (HP series II, model 5890, USA). Total number of bacteria and MPN of cellulolytic bacteria were estimated with 10^{-7} to 10^{-9} dilutions of the cultures at in vitro 24 h incubation, by counting the colony forming unit (c.f.u.ml^{-1}) grown in the roll tube of RGCMSA medium (Bryant and Burkey, 1953) and measuring the frequency of the breakdown of filter paper (Whatman No.1) in the Dehority medium, respectively.

Crude protein was determined according to the procedure of A.O.A.C. (1990), and neutral detergent fiber (NDF) and acid detergent fiber (ADF) were according to methods of Van Soest et al. (1991) without the use of sodium sulfite and amylase. Acid detergent lignin (ADL) was determined using 72% H_2SO_4 solution as modified by Van Soest et al. (1991).

Statistical analysis

Data was analyzed using an analysis of variance, and means were separated by the Turkey-Kramer test with the F-test significant at the 0.05 probability level (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Both pure and rumen mixed cultures almost showed positive responses of activities of forage degradation and microbial growth, with carbon sources of barley grain and orchardgrass hay (Table 1). The growth of *S. bovis*, *S. ruminantium*, *P. ruminicola*, *M. elsidenii* and *B. fibrisolvens* were significantly higher ($p<0.05$) at any concentration of Tween 80 than the control, with the most of the bacteria showing the distinct response at earlier incubation stages (Table 2). Among the cellulolytic bacteria *F. succinogenes* only showed the significant ($p<0.05$) increase of cell growth at 20 h incubation at two concentrations of Tween 80, whereas *R. albus* and *R. flavefaciens* were not at all changed.

This fact is consistent with a result of Madamwar et al.

Table 2. Effects of Tween 80 on the growth on the in vitro incubation of ruminal pure cultures.

Time after the inoculation (h)	Bacterial proteins (μg ml^{-1})				
	4 h	8 h	12 h	16 h	20 h
Streptococcus bovis					
Control (none)	33.9^{a}	90.9^{a}	87.2^{a}	75.3^{a}	68.1^{a}
TW 80-0.05%	105.2^{b}	122.5^{b}	102.5^{b}	83.8^{b}	104.6^{b}
TW 80-0.1%	110.6^{b}	117.5^{b}	105.9^{b}	94.4^{c}	103.8^{b}
SE	42.8	45.8	10.0	9.6	20.8
Prevotella ruminicola					
Control (none)	trace	8.1^{a}	6.9^{a}	7.8^{a}	9.3^{a}
TW 80-0.05%	trace	12.5^{b}	13.5^{b}	11.8^{b}	11.1^{a}
TW 80-0.1%	12.6	13.2^{b}	15.3^{b}	10.6^{b}	14.5^{b}
SE	n.d	2.8	4.4	2.1	2.0
Megasphaera elsdinii					
Control (none)	trace	5.4^{a}	7.8^{a}	8.9^{a}	7.5^{a}
TW 80-0.05%	trace	16.2^{b}	19.1^{b}	13.0^{b}	15.1^{b}
TW 80-0.1%	trace	14.1^{b}	16.3^{b}	12.3^{b}	13.8^{b}
SE	n.d	5.7	5.9	2.2	4.1
Selenomonas ruminantium					
Control (none)	21.0^{a}	28.3^{a}	46.7^{a}	36.5^{a}	143.2^{a}
TW 80-0.05%	25.5^{a}	47.4^{b}	100.5^{c}	185.4^{b}	176.3^{b}
TW 80-0.1%	31.7^{b}	33.3^{a}	69.0^{b}	164.1^{b}	180.7^{b}
SE	5.0	8.0	11.3	59.4	26.1
Butyrivibrio fibrisolvens					
Control (none)	22.5^{a}	44.0^{a}	60.7^{a}	109.0^{a}	129.6^{a}
TW 80-0.05%	26.9^{a}	52.0^{b}	84.0^{c}	129.3^{b}	140.4^{b}
TW 80-0.1%	29.5^{b}	49.8^{b}	75.1^{b}	125.5^{b}	132.1^{a}
SE	3.5	4.1	19.5	6.5	5.8
Fibrobacta succinogenes					
Control (none)	7.8^{b}	12.1^{b}	11.5^{b}	75.3^{a}	45.9^{a}
TW 80-0.05%	5.4^{a}	9.2^{a}	4.5^{a}	73.4^{a}	158.5^{c}
TW 80-0.1%	8.8^{b}	8.9^{a}	5.9^{a}	88.6^{a}	97.5^{b}
SE	1.7	3.1	3.7	8.3	56.4
Ruminococcus albus					
Control (none)	10.6^{a}	47.6^{a}	100.8^{b}	287.8^{b}	385.6^{a}
TW 80-0.05%	14.9^{b}	54.1^{b}	86.9^{a}	205.6^{a}	380.0^{a}
TW 80-0.1%	11.4^{a}	57.9^{b}	97.3^{b}	262.5^{b}	382.8^{a}
SE	2.3	2.8	7.2	42.1	2.8
Ruminococcus flavefaciens					
Control (none)	7.7^{a}	16.7^{a}	16.6^{a}	20.4^{a}	36.5^{a}
TW 80-0.05%	8.4^{a}	19.4^{a}	21.7^{b}	31.6^{b}	40.2^{a}
TW 80- .1%	29.2^{b}	17.5^{a}	25.6^{b}	29.1^{b}	41.0^{a}
SE	12.2	1.4	4.5	5.9	2.1

TW 80-0.05% and TW 80-0.1% mean treatments of 0.05% and 0.1% concentrations of Tween 80.
Means with different superscripts within the same column differed significantly at $p<0.05$.
Each value in the table represents a mean of five replications.

(1991) who showed that some surfactants increased anaerobic digestion of water hyacinth-cattle dung with a maximum of more than 114% higher fermentation gas production.

Since enzyme activity can be increased by surfactants (Fendler and Fendler. 1975; Castanon and Wilke. 1981; Kim et al., 1982; Ooshima et al., 1986; Helle et al., 1993), the microorganisms examined may be provided a highly localized substrate. It was previously shown that a surfactant Tween 80 increases enzymatic degradation of fibrous forages, especially more degradable fractions of the plant (Goto et al., 2002). That surfactant also seems to increase the accessibility of enzymes to the substrate, as shown by water- and enzyme-holding capacities of the substrates. The rate and extent of the improvement of enzymatic degradation were thus suggested to depend on the interrelationship between the resistance of plant structure and inherent digestion ability of bacteria species in the rumen.

Table 3. Effects of Tween 80 on bacterial number, DMD and fermentation profiles on the *in vitro* incubation of rumen mixed culture.

Treatment	pH	DMD (g kg^{-1})	VFA (mmol g^{-1}DDM)	VFA (mmol %)						Cellulolytic bacteria (MPN ml^{-1})	Total bacterial number (c.f.u. ml^{-1})
				Acetate	Propionate	iso-Butyrate	n-Butyrate	iso-Valerate	n-Valerate		
Barley grain (24 h incubation)											
Control (none)	5.96^{b}	354.1^{a}	86.9^{a}	63.9^{a}	32.2^{a}	0.6^{a}	2.8^{a}	0.3^{b}	0.4^{a}	4.3×10^{5}	1.9×10^{9}
TW 80-0.01%	5.92^{b}	369.5^{b}	91.2^{ab}	65.6^{a}	30.6^{a}	0.8^{a}	2.9^{a}	0.3^{b}	0.4^{a}	2.3×10^{5}	2.5×10^{9}
TW 80-0.2%	5.79^{a}	395.7^{c}	93.0^{c}	61.7^{a}	34.5^{a}	0.8^{a}	2.6^{a}	0.1^{b}	0.3^{a}	2.3×10^{5}	2.3×10^{9}
SE	0.09	6.4	3.1	1.9	1.9	0.1	0.1	0.1	0.1	-	-
Orchardgrass hay (48 h incubation)											
Control (none)	6.73^{a}	534.4^{a}	140.1^{a}	72.5^{b}	24.2^{a}	1.2^{a}	1.5^{a}	0.2^{a}	0.5^{a}	3.9×10^{8}	1.1×10^{9}
TW 80-0.01%	6.73^{a}	519.5^{a}	157.5^{ab}	70.9^{b}	25.9^{a}	1.2^{a}	1.4^{a}	0.2^{a}	0.4^{a}	7.5×10^{7}	1.3×10^{9}
TW 80-0.2%	6.71^{a}	585.6^{b}	185.7^{b}	63.6^{a}	33.3^{b}	1.2^{a}	1.3^{a}	0.2^{a}	0.4^{a}	4.3×10^{8}	1.3×10^{9}
SE	0.01	18.9	23.1	4.7	4.8	<0.01	0.1	<0.01	0.1	-	-

Means with different superscripts within the same column in the same substrate differed significantly at $p<0.05$.
Each value in the table represents a mean of five replications.
DMD, dry matter degradability; VFA, volatile fatty acids; MPN, most probable number; c.f.u., colony forming unit.

Therefore, the greater response of the growth of five non-cellulolytic bacteria compared to the cellulolytic bacteria would contribute to the higher degradation of barley grain through the relatively higher swelling capability of granular starch. Aksenova et al. (1994) reported that presowing seed treatment with Tween 80 enhanced water adsorption and germination of seeds of winter wheat, especially in less drought-resistant cultivars. It was also certain that F. succinogenes can be enhanced with the presence of Tween 80 since the bacterium possesses greater enzymatic activities of cellulases as compared to the two other fiber-degrading bacteria examined in this study (Yan et al., 1997). It is not however known in this study whether such enhancement of microbial growth is related to some supplementary nutritional effects of Tween 80 on rumen microorganisms.

Rumen mixed culture also showed positive response of the fermentation profiles, although it varied depending on concentrations of Tween 80 and substrates (Table 3). The pH value of the *in vitro* incubation of barley grain was significantly lowered ($p<0.05$) at a 0.2% concentration of Tween 80 than the control (none) and 0.01% Tween 80. The DMD of barley grain and orchardgrass hay was also significantly higher ($p<0.05$) at a 0.2% concentration of Tween 80 than the corresponding of the two other concentrations. The increased swelling of the substrates would contribute to the improvement of their enzyme accessibility and DMD, especially orchardgrass hay, since the MPN of cellulolytic bacteria was not changed in agreement with the result of *R.albus* and *R.flavefacienes*.

Total VFA production (mmol g^{-1}DDM) of barley grain and orchardgrass hay was significantly ($P<0.05$) increased by 0.2% concentration of Tween 80, while those of treatment of 0.01% concentration were intermediate between treatments of none and 0.2% concentrations. Since treatment of 0.2% Tween 80 increased DMD of both substrates, the higher efficiency of VFA conversion would be therefore associated with the significantly ($p<0.05$) higher ratio of propionate to acetate observed with orchardgrass hay. The same tendency of ratio of propionate to acetate was also observed with barley grain. Thus, it was certain that such drastic change in the VFA composition was resulted from increased activities of propionate-forming bacteria such as *S. ruminantium*, *B. fibrisolvens* and *M. elsidenii*, since those can be affected by the surfactant.

Ionophore is known to increase propionate and decrease acetate production in the rumen (Hino et al., 1994; Duff et al., 1995). Propionate leads to decrease methane production from rumen resulting in increased feed efficiency, which can be associated with reduced activities of rumen protozoa and fiber-degrading bacteria. Therefore, the former effect is apparently the same with effect of Tween 80, indicating the possibility of applying this surfactant to beef cattle production where the meat can be efficiently converted from propionate produced in the rumen. It is also interested to clarify effect of Tween 80 on the reduction of methane production from animal industry.

CONCLUSION

The result of this study suggests that Tween 80 increases forage degradability and microbial growth and flora in the rumen as well as propionate production. However, further research is needed to elucidate effects of Tween 80 on gas composition and production by rumen microorganisms with various feedstuffs. Possibility of utilization of Tween 80 alone or in combination with antibiotic such as salinomycine, in order to improve the fattening efficiency of beef production and reduction of methane emission also needs to be studied.

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