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Seasonality of fibrolytic enzyme activity in herbivore microbial ecosystems

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Fibre (cellulose, hemicellulose and lignin) is the most abundant polysaccharide in nature and is hydrolysed by gut micro-organisms of herbivores because they can produce a set of extracellular enzymes. This study examined seasonal changes in the fibrolytic enzyme activity of microbial ecosystems of five herbivores (buffalo, cow, impala, wildebeest and zebra). Crude protein extracts obtained from the aforestated ecosystems were assayed for exocellulase, endocellulase, cellobiase and xylanase by incubating with crystalline cellulose, carboxymethylcellulose, p-nitrophenyl ß-1, 4-D-gulcopyranoside and xylan at optimum pH (5.5 to 6.5) for 1, 2, and 48 h, respectively. The specific activities (µg reducing sugar/mg crude protein) of all enzymes varied ($p<0.001$) among ecosystems and between seasons. Generally, the exocellulase specific activities in all ecosystems increased from summer to winter whilst the specific activities of endocellulase and xylanase decreased. The cellobiase activity decreased for buffalo and impala but increased for the others. It is only the zebra that showed the most superiority to the cow for all enzyme systems. These results suggest that *in vitro* digestion of fibre would depend on the season the ecosystem is collected and the source of the ecosystem. Microbial ecosystem from the zebra is one with the highest activity that could benefit the ruminant production system.

Key words: Seasonality, herbivores, microbial ecosystems, enzymes.

INTRODUCTION

In natural pastures, herbivores browse and graze on forages that are rich in fibre while domesticated livestock depends, partly, on fibrous feeds (hay, fibrous crop residues and legumes) and supplementation. Abreu (1994) reported that more than half of the fibrous feeds in the European countries eaten by animals are wasted as faeces; it could even be higher in Africa where a) feeds are poorly supplemented with protein sources and b) the lignin content of grasses from the tropics are higher than those harvested from temperate regions reducing cellulose accessible to microbial fermentation (Van Soest, 1982). High faecal waste is due to the limited ability of the herbivore microflora to hydrolize and to utilize fibre. Consequently, a plethora of studies focusing on pure cultures of fibrolytic microorganisms (Varel et al., 1991), mixed cultures of fibrolytic micro-organisms (Colombatto et al., 2003), components of cellulase enzymes (Beldman et al., 1985) and unfractionated enzyme systems (Lynd et al., 2002) have been conducted to increase our understanding of fibrolysis. An active and diverse microflora (bacteria, fungi and protozoan) is responsible for the breakdown in both fore- and hindgut of herbivores. Although bacteria (Flint, 1994), fungi (Fonty and Gouet, 1994) and protozoans (Jouany and Ushida, 1994) have been shown to exhibit cellulolytic activities, not all species living in the fore- and hindgut are fibrolytic (Chen and Weimer, 2001).

Microbes digest fibre by expressing three major enzymes, exocellulases, endocellulases and cellobiases

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which act synergistically on the substrate to release soluble sugars (Gilbert and Hazlewood, 1993). These soluble sugars are ingested and fermented by microbes liberating end-products such as volatile fatty acids. Cellulase enzyme systems are often more complex due to the nature of cellulose substrate, which is made up of heterogeneous intertwined polysaccharide chains with varying degrees of crystallinity, hemicelluloses and pectins embedded in lignin. Therefore, an efficient cellulase enzyme system would require hemicellulase, pectinase, and ligninase to expose the embedded cellulose. Supplementation of ruminant diets with exogenous fibrolytic enzymes has been shown to improve animal performance and milk production (Beauchemin et al., 2003). It is also possible that enzyme expression is affected when cellulolytic microbial population is maintained by supplementing ruminant feeds with concentrates (Tafaj et al., 2001), protein, nitrogen (Yang, 2002) and phosphorus.

Others (Russel and Hespell, 1981; Bergen et al., 1982) showed that the growth rate of bacteria is partially a function of the availability of substrate while Cassia et al. (1999) showed that the growth rate of endoglucanases was higher in cellobiose fed cultures, but its production was greater in cultures grown on amorphous cellulose. Although microbes (*Aspergillus japonicus, Aspergillus oryzae, Aspergillus niger (MTCC 3496), Bacillus licheniformis, Tricoderma* and *Fibrobacter succinogenes S85*) producing enzyme products, in many cases, are similar, the types and activity of enzymes produced can vary widely depending on the strain selected, the growth substrate and culture condition used. Seasonal changes are associated with the variation in ingredient composition and thus fibre composition (Abreu et al., 1994) of the selected diet. In Africa, the long dry season (winter) is characterized by maturity and wilting of grass, inadequacy and poor nutritive value of forages (Sundset et al., 2009; Nicholson, 1984) while summers are characterized by the availability of grazing pastures of higher qualities. In winter, these animals suffer severe nutritional stresses due to poor nutritional value of pastures and shortages in supply. Strategies enhancing the unlocking of energy from cellulose rich forages, especially in winter are vital. Browsing different ecosystems using 16S rRNA gene sequence showed more bacteria species that were previously identified with a culture-based method (Karasov et al., 2011; Naya and Karasov, 2011; Karasov and Carey, 2009). It has also been noticed that analyses of new fermentative chambers always elucidate new operational taxonomic units (Naya and Karasov, 2011). Therefore, browsing new ecosystems especially in the wild, and grazing on veld, may elucidate microbes of higher fibrolytic potential.

Most studies on enzyme systems focus on isolated specific microbial strains (Matulova et al., 2005; Weimer and Weston, 1985) while a few studies have sampled enzymes in rumen fluid (Smith et al., 1973). The rate and extent of fibre digestion in the rumen are largely dependent on the population size of cellulolytic bacteria that indirectly affects enzyme concentration. Studies specifying appropriate seasons for enzyme sampling are limited (Koike et al., 2000). This study will determine the specific activity of fibrolytic enzymes (exocellulase, endocellulase, cellobiase and xylanase) from five herbivores (buffalo, cow, impala, wildebeest and zebra). Secondly, this study will relate these specific activities to enzyme concentrations in order to deduce the relative abundance of each enzyme in the cellulase enzyme system, with the aim of determining the appropriate season to sample microbial ecosystems for fibrolytic enzyme assays. Specific activities of herbivores from the wild will be compared with domesticated cows, to ascertain if they could be of any potential benefit for ruminant farm animals.

MATERIALS AND METHODS

Cellulase from *A. niger*, cellobiase (Novozyme®), and xylanase from *Thermomyces ianuginosus* (Novozyme®) purchased from Sigma (USA) were the commercial enzymes utilized in this study. The substrates were carboxylmethyl cellulose sodium salt (CMC) from FLUKA Bichemica (Germany), crystalline cellulose (powder) from ALDRICH® (Germany), p-nitrophenyl β-D-glucopyranoside (pNP-G), and xylan from beech wood (high grade) from Sigma (USA). Phenylmethylsulfonyl fluoride (PMSF), D-(+)-xylose, D-(+)-glucose and phenol were chemicals from Sigma (USA). Polyethylene glycol 20 000 (PEG 20 000) was from MERK Laboratory supplies, South Africa, Micro BCA™ protein assay kit was from PIERCE (USA) and 3,5-Dinitrosalicylic was from FLUKA (Switzerland). All the other common chemicals such as glacial acetic acid, sodium azide and ethylene diamine tetra acetic acid (EDTA) were bought locally from Capital Supplies, South Africa. Dialysis tubing cellulose membrane (10 000 molecular weight cut-off) was from Sigma-Aldrich. All spectral scans were carried out with Virian Scan 50® Bio UV-Visible spectrophotometer from Varian Australia Pty (Ltd), Australia.

Samples were collected from animal species with no preference to sex in December (summer) and in May (winter). *Bos taurus* (fistulated jersey cows) was from the Ukilinga Research farm, University of KwaZulu Natal, Pietermaritzburg. *Equus quagga boehmi* (zebra), *Connochaetes taurinus albojubatus* (wildebeest), *Aepyceros melampus* (impala) and *Syncerus caffer* (buffalo) were from Tala Game Reserve, Umbumbulu, KwaZulu-Natal (SA). Game animals such as the impala, wildebeest, zebra and buffalo were grazing on a dry land in an open field where *Pennisetum clandestinum* (Kikuyu grass) standing hay and other fibres were dominant. The buffalo often migrated to the valleys where they consumed standing hay, while the impala selected green material from the pasture. Unfortunately, we did not determine the diet composition. At the Ukulinga research farm, fistulated cows were fed entirely on hay. Rumen digesta (200 ml) was collected from cows, strained through four layers of cheese cloth as described by Smith et al. (1974), and treated immediately with 150 µl of phyenylmethylsulfonyl fluoride (0.1 mM PMSF) to inhibit proteases from lysing enzymes (Owolabi et al., 1988).

On the other hand, faeces were collected *in situ* (less than 2 min after defecation) from the other herbivores (buffalo, impala, wildebeest, and zebra) and placed immediately in an airtight thermo

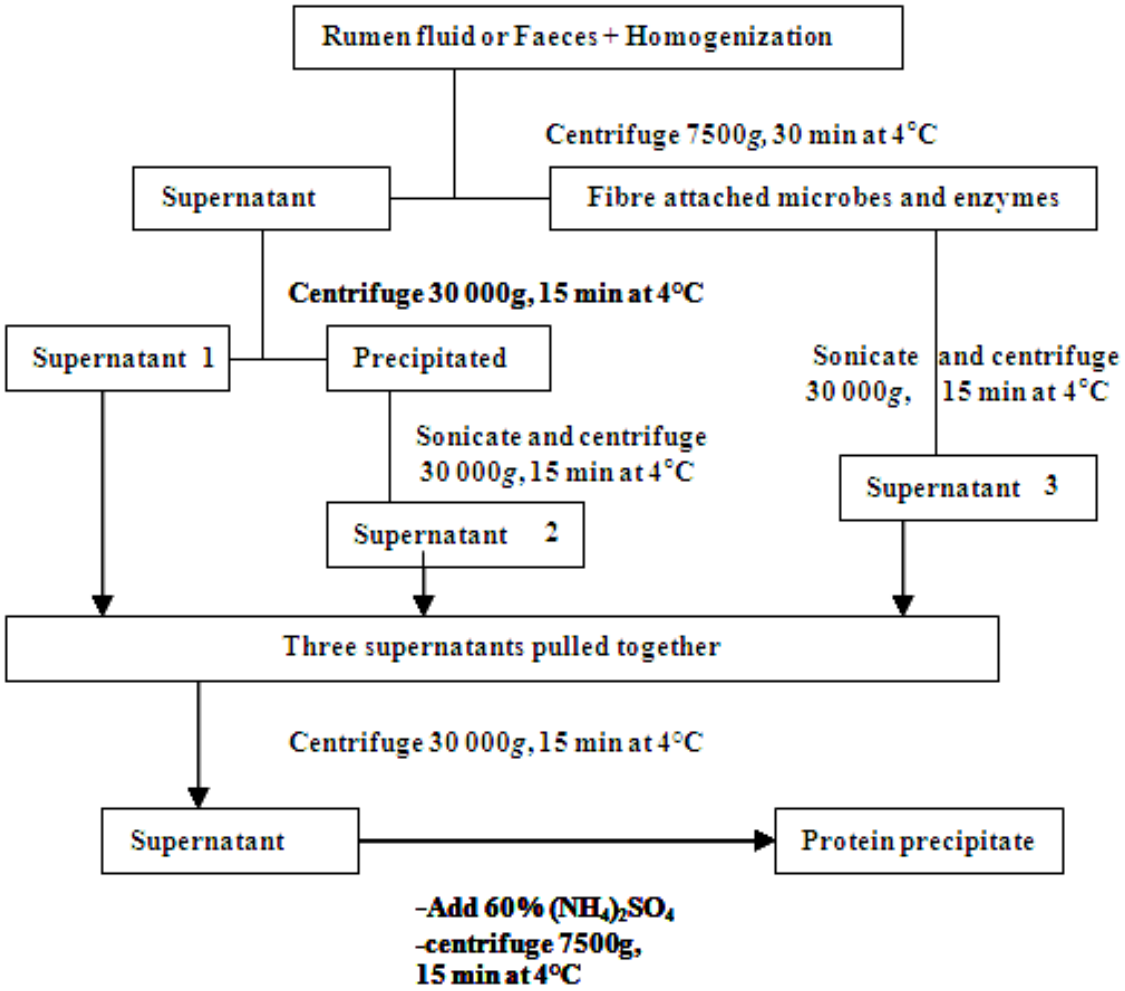

Figure 1. A flow chart of the precipitation of cellulases and hemicellulases from rumen fluid and faeces. Crude proteins were precipitated from 100 ml of rumen fluid or 100 ml of faecal fluid (made by mixing 100 g faeces and 100 ml buffer before squeezing through four layers of cheese cloth) by 60% ammonium sulphate.

flask maintained at 38°C. To maintain anaerobiosis in the flask, CO_2 was produced by reacting $CaCO_3$ with 50% HCl. Prior to further processing, faeces (100 g) was diluted with a buffer solution (100 ml of 50 mM sodium acetate (pH 5.5) containing 0.02% NaN_3 and 1 mM EDTA) and treated immediately with 150 µl of phyenylmethylsulfonyl fluoride (0.1 mM PMSF). Crude protein extracts were isolated following the procedure described by Henry et al. (1974) (Figure 1). With the aid of a pipette, each sample solution was transferred into a dialysis membrane and immersed in a 2 L storage buffer solution overnight (12 h), and then was concentrated using polyethylene glycol 20 000. The Micro BCA™ Protein assay kit (Richardson, 2000) was used to prepare a standard curve for determining crude protein concentration. Exocellulase was assayed by pipetting 0.5 ml of 1% (m/v) crystalline cellulose in the reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0) into 0.5 ml of crude protein solution obtained from rumen fluid or faeces and incubated for 48 h at 38°C following the method described by Gerrit et al. (1984).

The activity of endocellulase was determined by reacting 0.5 ml of 0.5% (m/v) carboxymethyl cellulose (amorphous cellulose) in the reaction buffer with 0.5 ml of crude protein solution obtained from rumen fluid or faeces and incubated for 2 h at 38°C following the method described by Gerrit et al. (1984). Cellobiase activity was measured in a mixture of 0.5 ml of 0.1% (m/v) *p*NP-G in the reaction buffer and 0.5 ml of crude protein solution and incubated for 1 h, based on the method described by Frutos et al. (2002). Xylanase hydrolyses xylan into xylose and oligosaccharides (Chivero et al., 2001) and was assayed according to the procedure described by Seyis and Aksoz (2005). Xylan [0.6 ml of 0.1% (m/v) xylan solution in the reaction buffer] was pipetted into 0.4 ml of crude protein solution and incubated at 38°C for 2 h. Reducing sugars in sample solutions were determined using the dinitro-salicylic (DNS) method (Miller, 1959).

All enzyme assay reactions were stopped by boiling for 5 min before analyzing for the reducing sugars after centrifugation (6000 x *g* for 5 min). Each ecosystem was represented by three samples, each of which was analyzed in triplicates for the different seasons. Specific activity was defined as µg of reducing sugar/ mg crude protein. The effects of ecosystem and season were determined using a two way (ecosystem and species) analysis of variance. This model was used to estimate the variance components with and

Table 1. Seasonal variation of endocellulase and exocellulase specific activities of crude protein extracts from herbivore microbial ecosystems.

Enzyme activity	Endocellulase specific activity (µg glucose/mg)		Exocellulase specific activity (µg glucose/mg)	
Substrate	Carboxylmethyl cellulose		Crystalline cellulose	
	Summer	Winter	Summer	Winter
Cow	1596[b]	704[c]	573[c]	881[d]
Buffalo	985[c]	680[c]	749[b]	841[d]
Impala	412[d]	629[d]	789[a]	1097[c]
Wildebeest	470[d]	893[b]	746[b]	1512[b]
Zebra	3048[a]	1434[a]	775[a]	2108[a]
SEM with cow (df=20)	48.6		51.26	
SEM without cow (df=16)	54.1		56.7	
p<	0.0001		0.0001	

[a, b, c, d, e] Numbers under same enzyme with different superscripts were significantly different.

without the cow's data. The analysis including the cow's data are reported because all the effects were consistently significant, at least at the 5% level of probability.

RESULTS AND DISCUSSION

Exocellulase specific activity differed ($p<0.0001$) between seasons and among the sampled herbivore microbial ecosystems (Table 1 and Supplementary Table 1). The activity of this enzyme increased from summer to winter in all other ecosystems by 1.12 to 3.33 folds. The impala, buffalo, cow and wildebeest had the lowest improvements in exocellulase concentrations while the zebra had the highest. Endocellulase specific activity differed ($p<0.0001$) among the sampled herbivore microbial ecosystems and between seasons, with the overall effect that the endocellulase activity was lower in winter than in summer for all, except the impala and wildebeest microbial ecosystems (Table 1 and Appendix 1). Among species, endocellulase specific activity in summer was highest in the zebra, intermediate in the cow and lowest in the buffalo, impala and wildebeest.

The xylanase (hemicellulase) specific activity differed ($p< 0.0001$) among the sampled herbivore microbial ecosystems and between seasons (Table 2 and Appendix 1). The zebra recorded the highest specific activity. The cow's system had intermediate activities, while impala, buffalo and wildebeest recorded the lowest xylanase activity. The xylanase specific activity increased noticeably in summer for the zebra ecosystem, and slightly for impala and cow. A significant variation in cellobiase specific activity was observed among animal species and between seasons ($p<0.0001$; Table 2 and Supplementary Table 1). Apart from the high variation of cellobiase specific activity observed in the zebra, the rest of the animals responded minimally to seasonal changes. Among herbivore species, the zebra recorded the highest enzyme specific activities, while the rest of the animals were relatively similar in cellobiase specific activities.

This comparative study used the specific activity per unit protein to estimate the variation of four fibrolytic enzymes concentrations (Bruce et al., 1984), though recognizing that microbes contribute much more (Stevens and Hume, 1998). These seasonal and animal variation in specific activities of crude protein extracts of these ecosystems could be attributed to changes in type and quality of available forage which directly affects the nutrient composition of selected diets, diet's ability to meet microbial requirements for various nutrients (El-Shazly et al., 1961; Scott and Dehority, 1965) and can elicit changes in the composition of microbial population, voluntary intake of forage by herbivores and the efficiency of different fermentation chambers (Kamra, 2005).

Onset of summers are characterized by an abundance of young and (or) green forage while the winters are characterized by mature, sun-cured grasses and green shrubby trees. The degree to which these changes occur is modulated by environmental stresses such as high temperatures (Van Soest, 1988) and infertile soils (Roberts, 1987). Consequently, forages selected and consumed by herbivores are richer in crystalline cellulose and poorer in amorphous cellulose in winter than in summer. Given that changes in microbial population are associated with the availability of substrate for microbial metabolism, it would be expected for animals foraging natural pasture that the activities of exocellulase and endocellulase would correlate with substrate availability. This agrees with our observation indicating high endocellulase activity in summer and exocellulase activity in winter. The high concentrations of xylanase in summer could also be due to the richness of soluble

Table 2. Seasonal variation of xylanase and cellobiase specific activities of crude protein extracts from herbivore microbial ecosystems.

Enzyme activity	Xylanase specific activity (µg xylose/mg)		Cellobiase specific activity (µg glucose/mg)	
Substrate	Xylan		cellobiose	
	Summer	Winter	summer	winter
Cow	6005[b]	5312[a]	64.8[b]	34.3[c]
Buffalo	812[e]	1355[b]	8.0[e]	18.7[d]
Impala	2489[c]	1742[b]	37.3 [c]	29.2[c]
Wildebeest	1256[d]	1402[b]	29.3 [d]	67.6[b]
Zebra	21811[a]	6031[a]	327.0[a]	90.5[a]
SEM with cow (df=20)	246.2		3.71	
SEM without cow (df=16)	223.1		3.92	
p<	0.0001		0.0001	

[a, b, c, d, e]Numbers under same enzyme with different superscripts were significantly different.

carbohydrates (amorphous cellulose and hemicelluloses) in young and vegetative forages which naturally would decrease in winter. Intake of predominantly fibre rich forages in winter and/or prior intestinal digestion (in a zebra) allows cellulose to be the most prevalent and available microbial substrates in winter which is associated with an increase in fibrolytic enzymes concentration. The aforestated inference concurs with previous studies demonstrating positive influences of substrate availability on microbial population (Waldo, 1972; Van Soest, 1973; Weimer et al., 1990).

It is known that ruminants retain fibrous feeds in the rumen for extended periods until the fibre has been reduced to lengths and sizes that facilitate passage through the reticulo-omasal orifice. During the period of residence in the rumen, the fibre undergoes microbial breakdown such that most of the available fibre is fermented by rumen microbes. It is possible that the prolonged retention of fibre in the rumen can reduce the need for microbial adaptation of the fibrolytic enzyme system to achieve rapid fibrolysis. In view of the attendant extensive fibrolysis in the rumen, digesta reaching the caeco-colon is poor in fermentable fibre though enriched with endogenous substances bearing microbial growth factors. In sharp contrast, whilst microbial growth factors exist in digesta entering the caeco-colon of non-ruminant herbivores, fibre contained therein was rich in fermentable fibre which, in addition, has been altered as a result of physical and chemical processes (chewing, reaction with acid and alkali) occurring at the anterior part of the digestive tract. It is perhaps for these differences that non-ruminant herbivores for example zebra exhibited higher activity for all fibrolytic enzymes than ruminants (wildebeest, Buffalo, Impala). Furthermore, since digesta generally spends a very short time in the caeco-colon, it is possible that microbes in this organ have evolved efficient enzyme systems for hydrolysing fibre rapidly as a way of acquiring nutrients for growth and ensuring their survival. Studies are required to confirm or refute this postulation.

For cattle, it is noticeable that the activities of endo-cellulase, cellobiase and xylanase enzymes increased 3.4, 1.88 and 1.13 folds respectively in summer in the rumen. In view of summer increases in the activity of these enzymes, it is possible that amorphous cellulose, hemicellulose and cellobiose are largely fermented in the rumen with barely limited quantities escaping and reaching the caeco-colon. Assuming that superior activity of these enzymes in summer is typical of most ruminants on natural pasture, it could well be that the inferior activities of these enzymes in the faeces of buffalo, wildebeest and impala are justified by the limited substrate reaching the caeco-colon. Confounding this explanation is the summer increase in endocellulase activity in the buffalo, and xylanase and cellobiase activities in the impala which are difficult to explain. It is possible that there is differential diet selection amongst these herbivores.

When animals select fibre-rich forages whether in winter or summer, concentrations of fibre degrading enzymes are bound to increase relatively. This implies that the concentration of fibre degrading enzymes will be much lower in an animal in winter grazing on very young shoots or entirely green leaves in the pasture than in those that are fed with hay (such as the cow). In an unpublished work in this laboratory, the concentration of crystalline cellulases observed in supplemented sheep did not vary between winter and summer. It is possible that supplementation of hay with concentrates in the latter study might have altered the overall microbial fermentation resulting to a decreased cellulolytic enzyme expression. This observation agrees with others (Bergen

et al., 1982; Hiltner and Dehority, 1983) showing increased endoglucanase concentrations when carboxy-methyl cellulose was supplemented with concentrates. Supplementation of feed possibly increased the overall rate of expression but not the relative abundance of endocellulase as compared to the expression on pure carboxymethyl cellulose. Nsahlai and Umunna (1996) showed that by using *in-vitro* digestibility of a range of roughage diets, inoculum based on reconstituted sheep faeces relative to rumen inoculum underestimated gas production and *in vitro* dry matter digestibility. This study shows remarkable differences between enzymes activities of microbial ecosystems derived from the faeces of ruminant and non-ruminant herbivores. Seasonality of enzyme activity which is linked to the type of available feed, could even be more severe for non-ruminant than for ruminant herbivores when the ecosystems are derived from faeces. This can also introduce further variation in the results of *in vitro* studies based on faeces inoculums.

Results presented in this paper reveal remarkable variation in the activities of fibrolytic enzymes which is ascribed to the seasonality of type and quality of herbage and to the source of herbivore microbial ecosystems. These results suggest that the degree of *in vitro* digestion of fibre would depend on the season the microbial ecosystem is collected and on the source of the ecosystem. The hydrolysis of crystalline cellulose was much higher in winter than in summer and was higher in zebra than in the other species, whilst the specific activity of the rest of the enzymes was higher in summer than in winter and the zebra was still the best system and could benefit the farm ruminants. Microbial ecosystem from the zebra is one with the highest activity that could benefit the ruminant production system.

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Supplementary Table 1. Analyses of variance table of enzyme activity.

Exocellulase activity					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Model	9	0.70226680	0.07802964	166.94	<0.0001
Error	20	0.00934800	0.00046740		
Corrected Total	29	0.71161480			
Source	**DF**	**Type III SS**	**Mean Square**	**F Value**	**Pr > F**
Season	1	0.28460280	0.28460280	608.91	<0.0001
Animal	4	0.23968913	0.05992228	128.20	<0.0001
Season*Animal	4	0.17797487	0.04449372	95.19	<0.0001
Endocellulase activity					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Model	9	1463.246499	162.582944	661.79	<0.0001
Error	20	4.913399	0.245670		
Corrected Total	29	1468.159898			
Source	**DF**	**Type III SS**	**Mean Square**	**F Value**	**Pr > F**
Season	1	184.0510083	184.0510083	749.18	<0.0001
Animal	4	860.6944183	215.1736046	875.86	<0.0001
Season*Animal	4	418.5010722	104.6252680	425.88	<0.0001
Xylanase activity					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Model	9	299341.6865	33260.1874	1316.53	<0.0001
Error	20	505.2712	25.2636		
Corrected Total	29	299846.9576			
Source	**DF**	**Type III SS**	**Mean Square**	**F Value**	**Pr > F**
Season	1	22840.0506	22840.0506	904.07	<0.0001
Animal	4	195011.9310	48752.9827	1929.78	<0.0001
Season*Animal	4	81489.7049	20372.4262	806.40	<0.0001
Cellobiase activity					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Model	9	16.38204830	1.82022759	1283.54	<0.0001
Error	20	0.02836267	0.00141813		
Corrected Total	29	16.41041097			
Source	**DF**	**Type III SS**	**Mean Square**	**F Value**	**Pr > F**
Season	1	1.08186030	1.08186030	762.88	<0.0001
Animal	4	10.27484713	2.56871178	1811.33	<0.0001
Season*Animal	4	5.02534087	1.25633522	885.91	<0.0001