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CONTRASTING LEVELS OF FRUCTOSE AND UREA ADDED TO AN ANNUAL RYEGRASS BASED DIET: EFFECTS ON MICROBIAL PROTEIN SYNTHESIS, NUTRIENT DIGESTIBILITY AND FERMENTATION PARAMETERS IN CONTINUOUS CULTURE FERMENTERS

NIVELES CONTRASTANTES DE FRUCTOSA Y UREA AGREGADOS A UNA DIETA BASADA EN RAIGRÁS ANUAL: EFECTOS SOBRE SÍNTESIS DE PROTEÍNA MICROBIANA, DIGESTIBILIDAD DE NUTRIENTES Y PARÁMETROS DE FERMENTACIÓN EN FERMENTADORES DE FLUJO CONTINUO

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ABSTRACT

The objective of this experiment was to evaluate the effects of the addition of crystalline fructose and urea to an annual ryegrass-based diet on microbial protein synthesis, fermentation profile and nutrient apparent digestibility, using continuous culture fermenters. Six fermenters were used in a 3 x 2 factorial arrangement with three levels of water soluble carbohydrates (WSC) obtained by crystalline fructose addition (21, 24 and 27 g.100 g DM^{-1}; LWSC, MWSC and HWSC, respectively) and two levels of CP obtained by urea addition (14.6 and 18.6 g.100 g DM^{-1}, LCP and HCP, respectively). Four 10-d periods were ran sequentially (7-d for adaptation, 3-d for sampling). Microbial protein synthesis was assessed by purine to N ratio. There was a positive interaction between WSC and CP level on microbial protein synthesis (P<0.001). Water soluble carbohydrate level did not affect fermentation pH, ammonia concentration or total volatile fatty acids concentration (VFA). Greater CP levels also increased acetic acid proportion and tended to increase acetic to propionic acid ratio, whereas WSC level did not affect VFA proportions. Treatments did not affect nutrient digestibility. We conclude that the addition of crystalline fructose to annual ryegrass samples increased microbial protein synthesis at the greater levels of CP in diet.

KEY WORDS: annual ryegrass, continuous culture, crude protein, microbial protein synthesis, water soluble carbohydrate

RESUMEN

El objetivo de este experimento fue evaluar los efectos de la adición de fructosa cristalina y urea a una dieta basada en raigrás anual sobre la síntesis de proteína microbiana, la fermentación y la digestibilidad de los nutrientes, usando fermentadores de flujo continuo. Se usaron seis fermentadores de flujo continuo en un arreglo factorial 3x2, con tres niveles de hidratos de carbono solubles (WSC) obtenidos por la adición de fructosa cristalina (21, 24 y 27 g.100 g MS^{-1}; LWSC, MWSC y HWSC, respectivamente) y dos niveles de proteína bruta (CP) obtenidos por la adición de urea (14,6 y 18,6 g.100 g MS^{-1}, LCP y HCP, respectivamente). Se corrieron sucesivamente cuatro períodos de 10-d (7-d para adaptación, 3-d para muestreo). La síntesis de proteína microbiana se estimó por la relación purinas:N. Hubo una interacción significativa entre niveles de WSC y CP para síntesis de proteína microbiana (P<0,001). El nivel de WSC no afectó el pH, la concentración de amonio ni la concentración de ácidos grasos volátiles (VFA). Niveles más altos de CP aumentaron la proporción de ácido acético y tendieron a aumentar la relación acético propiónico, mientras que el nivel de WSC no afectó las proporciones de VFA. Los tratamientos no afectaron la digestibilidad de los nutrientes. Concluimos que la adición de fructosa cristalina a dietas basadas en raigrás anual aumentó la síntesis de proteína microbiana a los niveles más altos de CP en la dieta.

PALABRAS CLAVE: Raigrás anual, fermentadores de flujo continuo, proteína bruta, síntesis de proteína microbiana, hidratos de carbono solubles

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Introduction

Rumen microbes are able to synthetize microbial protein from non-protein N sources (Clark *et al*., 1992). For this synthesis to be efficient, water soluble carbohydrates (WSC) to CP ratio (WSC:CP) seems to be determinant (Johnson, 1976; Edwards *et al*., 2007; Hall & Huntington, 2008). Hoover and Stokes (1991) analyzed a range of NSC:RDP ratios within 2 and 10 and found that lower ratios led to higher microbial crude protein synthesis. However, high quality grasses (i.e., annual ryegrass, *Lolium multiflorum*) often can often show ratios lower than 2, from which little is known. This imbalance in turn leads to ammonia buildup in the rumen, which microorganisms cannot efficiently capture (Stern *et al*., 1978) reducing N use inefficiency (Da Silva *et al*., 2014).

Temperate grasses store energy in the form of fructans, commonly called water soluble carbohydrates (WSC) or sugars. Grass WSC:CP ratio can vary due to several factors (Parsons *et al*., 2004; Mayland *et al*., 2005; Gregorini *et al*., 2006; Moorby *et al*., 2006; Cosgrove *et al*., 2007). Fructans are readily available to microorganisms immediately after entering the rumen (Johnson, 1976), thus providing carbon skeletons and energy. Therefore, increased supply of WSC in diets high in non-protein N could improve N efficiency and microbial protein synthesis (Mansfield *et al*., 1994). In fact, Edwards *et al*. (2007) reviewed several research articles in dairy cattle and found that as WSC:CP ratio increases, the proportion of nitrogen lost by urine decreases. On the other hand, it is known that microbial protein synthesis depends partially on N supply. Most research shows that increasing true protein supply, in the form of polypeptides, oligopeptides or aminoacids, enhance microbial growth over ammonia (Hoover & Stokes, 1991), but little is known about the ability of microorganisms to capture inorganic N at contrasting levels of WSC supply. The objective of this experiment was to create contrasting WSC:CP ratios adding urea and fructose to an annual ryegrass based diet, to evaluate the effect on microbial protein synthesis, fermentation and digestibility

Material and Methods

Treatments

Six treatments were designed by a 3 x 2 factorial arrangement of treatments, with three levels of WSC (21, 24 and 27 g.100 g DM^{-1}; LWSC, MWSC and HWSC, low, medium and high WSC, respectively) and 2 levels of CP (14.6 and 18.6 g.100 g DM^{-1}, LCP and HCP, low and high CP, respectively); levels were designed to be within the possible range of annual ryegrass. A sample of dried (60ºC, 48 h) and ground (2-mm sieve) annual ryegrass (*Lolium multiflorum*, var Enhancer, Sucraseed, OR) harvested at the stage of flag leaf emergence was used as the basic feed to which crystalline fructose (Tate & Lyle, Decatur, IL, US, 99% purity) and ground urea were added (Table 1). Feed analysis included determinations of NDF (Van Soest *et al*., 1991), ADF (AOAC, 2000), lignin (Goering & Van Soest, 1970), CP (AOAC, 2000), soluble protein (Krishnamoorthy *et al*., 1982), degradable protein (Krishnamoorthy *et al*., 1983), WSC (Dubois *et al*., 1956; Hall, 2013), ether extract (AOAC, 2000), and starch content (Hall, 2009).

Continuous culture fermenters

The six dual-flow continuous culture fermenters used in this experiment were a modified version of the design described by Teather and Sauer (1988). Solid passage rate was set at 5%. h^{-1} and liquid dilution rate at 12%.h^{-1}, by regulating the buffer infusion pumps at 90 mL.h^{-1}. Each treatment was run for four 10-d periods sequentially (7-d for adaptation, 3-d for sampling). Whole rumen contents were taken from cannulated Holstein dairy cows fed a ration comprised of corn silage, grass hay and a grain-mix diet. All surgical and animal care protocols were approved by the Clemson University Animal Care and Use Committee (Protocol 2016-034). Liquid and solid ruminal contents were transported to the laboratory and homogenized in a blender while purged with CO_2. The blended mix was filtered through double layer cheesecloth and mixed with the buffer (Slyter *et al*., 1966) at a 1:1 ratio and added into the continuous culture fermenter vessel (approximately 750-mL total volume).

The temperature was kept at 39.5°C by a circulating heated water bath (Julabo, PA, USA). Fermenters were constantly purged with CO_2 (20 mL.min^{-1}). Fermenters were fed 20 g DM of the respective treatment per day at 0800 and 1600.

Sample collection and measurements

Overflow volume was recorded daily in refrigerated vessels. After measuring volume, a 10-mL sample of overflow was taken with a wide mouth (0.8 mm opening) pipette. This sample was used to estimate overflow DM content. Samples of overflow were kept to estimate NDF and ADF content, as well as to estimate the N to purine ratio and microbial protein synthesis (Zinn & Owens, 1986). Apparent DM, NDF and ADF digestibilities were estimated through simple weight differences between fed and outflow, while true DM digestibility was calculated subtracting bacterial DM outflow. Neutral detergent fiber and ADF content of the overflow were estimated in an ANKOM 2000 analyzer (Van Soest *et al*., 1991). Samples from overflow were treated to isolate a bacterial pellet in which to estimate the N to purine ratio, using differential centrifugation. Purine content in bacterial pellet and overflow was determined according to Zinn and Owens (1986).

Samples from the culture were collected at -2, 0, 2, 4, 6 and 8 h, with 0 h being the first daily feeding time at 0800. Culture pH (Hanna Instruments, Woonsocket, RI) was recorded at the same sampling times (plus an additional measurement at 1 h). A 4-ml sample was transferred into polycarbonate tubes containing 1-ml of 25g.100-ml^{-1} metaphosphoric acid. These samples were used to determine ammonia and VFA concentration. Ammonia concentration was estimated by colorimetric technique (Chaney & Marbach, 1962).

Volatile fatty acid concentrations were analyzed by gas chromatography with flame ionization detector on a Zebron ZB-FFAP 30 m x 0.25 mm x 0.25µm column (Phenomenex, Torrance, CA). The injection volume was 0.1 µl and samples were injected with a split ratio 10:1. Injector was kept at 270°C and detector at 250°C. The carrier gas was hydrogen at a flow rate of 26.9 mL.min^{-1}. Column oven temperature was programmed to increase from 120 to 150°C at a rate of 12°C.min^{-1}, and from 150 to 220°C at a rate of 20 °C.min^{-1}. Standard curves were run for each VFA using a standard VFA mix (Sigma-Aldrich VFA mix, PA, US) to estimate total VFA concentration (mM) as well as the molar proportions of individual VFA.

Table 1. Chemical composition of diets based on annual ryegrass differing in water soluble carbohydrates and crude protein content, fed to continuous culture fermenters

Tabla 1 Composición química de las dietas basadas en raygrás anual con diferentes contenidos de hidratos de carbono solubles y proteína bruta en fermentadores de flujo continuo.

g.100 g DM^{-1}	Diet					
	LWSC		MWSC		HWSC	
	LCP	HCP	LCP	HCP	LCP	HCP
CP	14.4	18.7	15.0	18.0	14.5	19.1
SP	5.2	9.3	5.4	9.4	5.0	9.6
RDP	9.8	14.0	10.2	13.7	9.8	14.5
NDF	49.2	49.0	47.4	46.5	45.4	46.1
ADF	30.2	29.5	29.3	29.2	28.9	30.8
ADL	2.9	4.2	2.5	2.9	2.5	2.4
WSC	21.2	21.0	24.1	24.5	27.1	26.9
EE	2.8	1.9	2.8	2.3	2.7	2.2
Starch	2.3	2.2	1.3	2.0	1.1	1.0

Water soluble carbohydrates: LWSC = 21 g WSC.100g DM^{-1}, MWSC: 24g WSC.100g DM^{-1}, HWSC 27 g WSC.100g DM^{-1}; LCP: 14.6g CP.100g DM^{-1}, HCP: 18.6g CP.100g DM^{-1}. CP= crude protein, SP= soluble protein, RDP= Ruminally degradable protein, a-NDF= neutral detergent fiber (residual ash included), ADF= acid detergent fiber, ADL= lignin (sa), WSC= water soluble carbohydrates, EE= ether extract.

Hidratos de carbono solubles: LWSC = 21g WSC.100g DM^{-1}, MWSC: 24g WSC.100g DM^{-1}, HWSC 27g WSC.100g DM^{-1}; LCP: 14,6g CP.100g DM^{-1}, HCP: 18,6g CP.100g DM^{-1}. CP= proteína bruta, SP= proteína soluble, RDP= Proteína degradable a nivel ruminal, a-NDF= Fibra detergente neutro incluyendo cenizas, ADF= fibra detergente ácido, ADL= lignina, WSC= hidratos de carbono solubles, EE= extracto etéreo

Statistical analyses

Dry matter, a-NDF and ADF digestibilities, as well as microbial protein synthesis data, were analyzed using the mixed procedure of SAS

(SAS Inst., Inc., Cary, NC) based on the following model: Yijk= μ + γi + πj + γπij + ρk + θl + εijkl, where Yijk is the observed value, μ is the overall mean, γi is the WSC effect (i= 1 to 3), πj is the CP effect (j= 1 to 2), γπij is the interaction between WSC and CP, ρk is the random effect of period (k = 1 to 4), θl is the random effect of fermenter, and εijkl is the experimental error. Ammonia, VFA, and pH data were analyzed with repeated measures using the mixed procedure of SAS (SAS Inst., Inc., Cary, NC). Linear and quadratic contrasts were used to evaluate the effect of WSC. Significance was determined at $P < 0.05$. Differences at $0.05 < P < 0.10$ were discussed as trends.

Results and Discussion

Fermentation parameters and nutrient digestibility

There was no effect ($P = 0.43$) of CP on average pH (6.28 vs 6.34, for LCP and HCP, respectively, Table 2). There was a tendency to a quadratic effect of WSC level on average pH, with MWSC resulting in the lowest values (Table 2). There was no interaction between WSC and CP level, or between WSC and sampling hour for pH ($P > 0.05$); however, there was an interaction between CP and sampling hour ($P = 0.003$, Figure 1). At 1 h and 2 h post-feeding, pH was greater in HCP than in LCP (Figure 1). On the other hand, HCP showed greater ammonia concentration (Table 2), because once in the rumen, urea is rapidly converted into ammonia. Ammonia level peaked in HCP at 2 h post-feeding and then slowly decreased, but always showed greater levels of ammonia than LCP (Figure 2). Greater concentrations of ammonia agreed with greater pH detected in HCP at 1 and 2 h post feeding, which reflects that urea had a buffering effect, as previously shown by Wanapat *et al*. (2009). On the other hand, WSC level had no effect on ammonia levels, which contrasts with the findings of Kim *et al*. (1999), who reported that

Table 2. Total and individual volatile fatty acids, ph and ammonia concentration in continuous culture fermenters fed annual ryegrass differing in water soluble carbohydrates and crude protein content

Tabla 2. Concentración de ácidos grasos volátiles totales e individuales, pH y amonio en fermentadores de flujo continuo alimentados con dietas basadas en raigrás anual variando el contenido de hidratos de carbono solubles y proteína bruta

	CP level		SEM	WSC level			SEM	*P*- value			
	LCP	HCP		LWSC	MWSC	HWSC		CP	WSC lin	WSC quad	WSC x CP
pH	6.27	6.34	0.07	6.38	6.21	6.32	0.05	NS	NS	**	NS
NH_4^+	5.95	13.82	0.70	10.17	9.44	10.04	0.74	**	NS	NS	NS
VFATotal mM (mM.100 mM^{-1})	36.86	39.22	2.65	34.84	41.57	37.71	2.46	NS	NS	**	NS
Acetic	46.01	50.49	0.79	50.04	47.02	47.70	1.22	**	NS	NS	NS
Propionic	26.17	24.97	0.86	24.90	25.82	25.99	1.02	NS	NS	NS	NS
Isobutyric	0.72	0.63	0.04	0.66	0.69	0.69	0.05	†	NS	NS	NS
Butyric	17.21	16.05	0.67	16.35	16.94	16.61	0.71	*	NS	NS	NS
Isovaleric	1.66	1.27	0.24	1.41	1.64	1.35	0.24	**	NS	*	NS
Valeric	5.62	4.44	0.42	4.38	5.28	5.42	0.49	*	†	NS	NS
A:P	1.78	2.04	0.07	2.03	1.84	1.86	0.10	†	NS	NS	NS

LWSC, MWSC and HWSC, low, medium and high water soluble carbohydrates, 21, 24 and 27g WSC.100g DM^{-1}. LCP and HCP, low and high crude protein, 14.6 and 18.6g.100g DM^{-1}. A:P= acetic to propionic ratio. SEM= Standard error mean. NS= not significant. Significance of the CP, WSC and their interactions were denoted by †=P<0.10, *=P<0.05, **=P<0.01

LWSC, MWSC and HWSC, nivel bajo, medio y alto de hidratos de carbono solubles, 21, 24 y 27g WSC.100g DM^{-1}. LCP y HCP, nivel bajo y alto de proteína bruta, 14,6 y 18,6g.100g MS^{-1}. A:P= relación acético:propiónico. SEM= error estándar de la media. NS= no significativo. La significancia de CP, WSC y sus interacciones se señala por: †=P<0,10, *=P<0,05, **=P<0,01

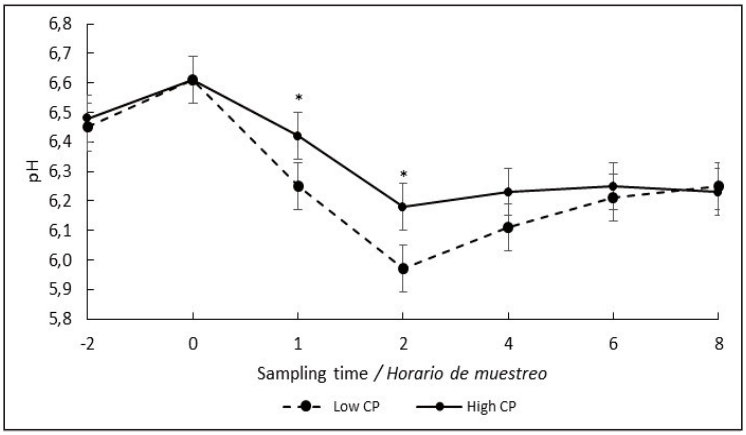

Figure 1. Continuous culture fermenters pH at different times post feeding resulting from diets containing low (14.6g.100g DM^{-1}) or high (18.6g.100g DM^{-1}) CP concentrations (sampling time x CP interaction, P = 0.003). Error bars = 0.0810, standard error of the mean. Asterisk indicates differences (P < 0.05) between diets within sampling time.

Figura 1. Promedio de pH diario en fermentadores de flujo continuo a diferentes horarios post alimentación, resultante de dietas conteniendo bajo (14,6g.100g MS^{-1}) y alto (18,6g.100g MS^{-1}) contenido de CP (interacción horario de muestreo x CP P= 0,003) . Barras de error= 0,0810, error estándar de la media. Los asteriscos indican diferencia significativas (P<0,05) entre medias.

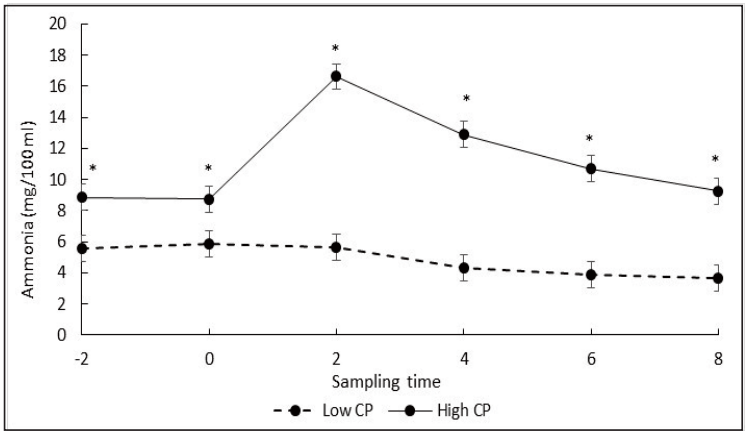

Figure 2. Continuous culture fermenters ammonia concentration at different times post feeding resulting from diets containing low (14.6g.100g DM^{-1}) or high (18.6g.100g DM^{-1}) CP concentrations. Error bars = 0.928, standard error of the mean. Asterisk indicates differences (P < 0.05) between treatment means.

Figura 2. Concentración de amonio en fermentadores de flujo continuo a diferentes tiempos post alimentación con dietas conteniendo bajo (14,6g.100g MS^{-1}) y alto (18,6g.100g MS^{-1}) contenido de CP. Barras de error= 0,928, error estándar de la media. Los asteriscos indican diferencia significativas (P<0,05) entre medias.

ruminal infusion of maltodextrin reduced rumen ammonia concentration and reduced the peak of ammonia concentration immediately after feeding.

Concentration of WSC resulted in a quadratic effect on total VFA concentration (P = 0.04, Table 2), the latter being greater in MWSC. This is consistent with the lower pH found in MWSC, since total VFA is an important determinant of pH (Dijkstra *et al*, 2012). The individual VFA molar proportions were not affected by WSC, except for a trend (P= 0.08) to a linear increase in valeric acid with increasing WSC level (Table 2). Contrastingly, Berthiaume *et al*. (2010) reported greater proportion of propionate and butyrate as well as lower proportion of acetate in cattle fed alfalfa with greater nonstructural carbohydrate content. Kim *et al*. (1999) reported greater molar proportion of butyric acid in maltodextrin supplemented cattle. However, in both studies, the treatments affected ruminal pH, thereby it is impossible to elucidate if the changes in VFA profile were due to substrate, pH or both.

On the other hand, CP level affected both acetic and butyric acid molar proportion (Table 2). The proportion of acetic acid was greater in HCP than in LCP, whereas the opposite occurred with butyric acid. Acetic to propionic acid ratio tended to be greater in HCP treatments (P= 0.06), even though no differences were detected in propionic acid concentration. Finally, HCP level also showed a lower molar proportion of isovaleric and valeric acid (Table 2). In a continuous culture experiment, Calsamiglia *et al*. (2008) analyzed the effect of both pH and diet on VFA concentration and concluded that pH affected

both acetate and butyrate concentrations. Culture pH explained 81% of the observed variation in acetate concentration, which increased 23.7 mM for each unit increase in pH. In the case of butyrate, pH explained 36% of the variation, being both factors negatively related. Even though the relation between VFA proportions and pH is more complex, it seems that culture pH had an effect affecting both acetate and buyrate proportion.

Neither WSC level nor CP level affected (Table 3) apparent DM digestibility (51.39g.100g DM^{-1}, on average), true DM digestibility (58.58g.100g DM^{-1}, on average), NDF digestibility (49.21g.100g DM^{-1}, on average) or ADF digestibility (40.87g.100g DM^{-1}, on average). Coincident with our findings, Mansfield *et al*. (1994) did not find significant effects of diet NSC concentration on DM digestibility. With respect to the effect of highly fermentable carbohydrates on fiber digestion, it has been studied previously (Calsamiglia *et al*., 2008) and it seems that those effects are also mediated by pH, which is one of the most important factors affecting fibrolytic bacteria activity (Russell and Wilson, 1996). Several researchers have shown that fiber digestibility is reduced when average pH is below 6.0 (Mouriño *et al*., 2001; Calsamiglia *et al*., 2008; Disjktra *et al*., 2012). Our average pH values were above 6.0 in all the treatments, which implies that the fermentation environment supported a good fiber fermentation even at the greater levels of WSC. On the other hand, the lack of effect of urea addition on DM and fiber digestibility coincides with reports by Stern *et al*. (1978).

Microbial protein synthesis

There was an interaction among CP and WSC level ($P<0.001$) on microbial protein synthesis (Figure 3). At the lower level of CP, there was no effect of WSC on microbial protein synthesis, whereas at the greater level of CP, increasing the level of WSC led to greater microbial protein synthesis (Figure 3). Kim *et al*. (1999) reported greater microbial protein synthesis when supplying maltodextrin either synchronized with protein supply or in a continuous infusion. Coincidently, Henning *et al*. (1991) found that a pulse dose of WSC at feeding time was the most effective way to increase microbial growth in batch culture. Forages under direct grazing sometime contain high concentration of soluble protein and an imbalance between WSC and highly soluble protein availability (Merry *et al*., 2006). This has led to the selection of forage varieties greater in WSC, which should lead to

Table 3. Dry matter, NDF and ADF digestibility of annual ryegrass differing in water soluble carbohydrates and crude protein content, fed to continuous culture fermenters

Tabla 3. Digestibilidad de la FDN, FDA y MS de raigrás anual variando su contenido de hidratos de carbono solubles y proteína bruta, en fermentadores de flujo continuo

g.100g DM^{-1}	CP level		SEM	WSC level			SEM	*P*- value			
	LCP	HCP		LWSC	MWSC	HWSC		CP	WSC lin	WSC qua	WSC x CP
App IVDMD	52.65	50.13	1.75	51.62	51.07	51.48	2.01	NS	NS	NS	NS
True IVDMD	58.61	58.55	1.31	58.29	58.04	59.42	1.60	NS	NS	NS	NS
NDFD	50.62	47.81	2.21	49.87	48.92	48.85	2.48	NS	NS	NS	NS
ADFD	42.03	39.72	2.59	44.01	40.66	37.94	3.22	NS	NS	NS	NS

Water soluble carbohydrates: LWSC = 21g.100g DM^{-1}, MWSC: 24g.100g DM^{-1}, HWSC 27g.100g DM^{-1}; CP (crude protein) content: LCP: 14.6g.100g DM^{-1}, HCP: 18.6g.100g DM^{-1}. IVDMD= in vitro dry matter digestibility, NDFD= neutral detergent fiber in vitro digestibility, ADFD= acid detergent fiber in vitro digestibility, WSC= water soluble carbohydrate main effect, CP = crude protein main effect, WSC x CP = water soluble carbohydrate x crude protein interaction. NS= not significant. Significance of the CP, WSC and their interactions were denoted by †=P<0.10, *=P<0.05, **=P<0.01

LWSC, MWSC and HWSC, nivel bajo, medio y alto de hidratos de carbono solubles, 21, 24 y 27g WSC.100g DM^{-1}. LCP y HCP, nivel bajo y alto de proteína bruta, 14,6 y 18,6g.100g MS^{-1}. IVDMD= digestibilidad in vitro de la materia seca, NDFD= digestibilidad in vitro de la FDN, ADFD= digestibilidad in vitro de la FDA, SEM= error estándar de la media. NS= no significativo. La significancia de CP, WSC y sus interacciones se señala por: †=P<0,10, *=P<0,05, **=P<0,01

improvements in N use efficiency. Berthiaume e*t al*. (2010) reported that high NSC alfalfa varieties increased the efficiency of N use by bacteria. Similarly, Merry *et al*. (2006), using high sugar ryegrass varieties in an in vitro system, found that the efficiency of N use was greater for high-sugar ryegrass silage than control. Additionally, in an in vivo experiment, high WSC concentration perennial ryegrass varieties led to lower rumen ammonia concentration, greater microbial N flows to the duodenum and greater efficiency of microbial protein synthesis (Merry *et al*., 2006). Once in the rumen, WSC (i.e., fructans and fructose) go quickly into solution and would therefore be available for rapid fermentation, yielding ATP and VFA that can later be used in combination with N sources in the synthesis of microbial protein (Johnson, 1976). That would explain the lower VFA concentration found in HWSC compared to MWSC, because part of the produced VFA would have been used for synthesis of bacterial aminoacids. Increasing forage WSC concentration, either by genetic improvement of varieties (Cosgrove *et al*., 2007)

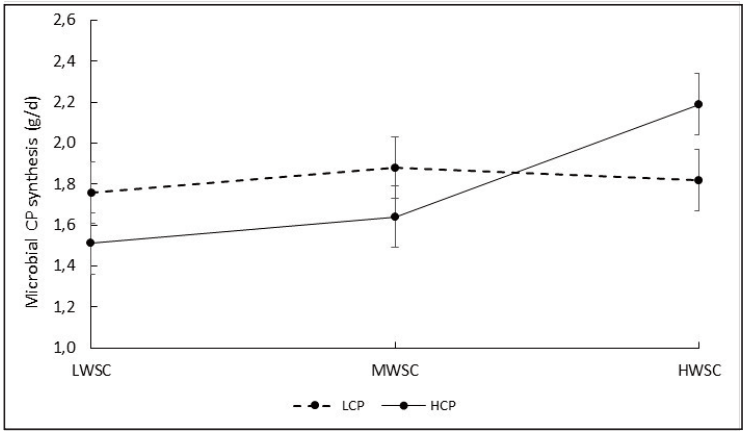

Figure 3. Microbial crude protein synthesis (g.d^{-1}) in continuous culture fermenters from diets containing low (14.6g.100g DM^{-1}) or high (18.6g.100g DM^{-1}) CP concentrations and low (LWSC= 21g.100g DM^{-1}), medium (MWSC= 24g.100g DM^{-1}) and high (HWSC= 27g.100g DM^{-1}) WSC content. Error bars = 0.150, standard error of the mean. Different letter means statistical differences (P< 0.05).

Figura 3. Síntesis de proteína microbiana (g.d^{-1}) en fermentadores de flujo continuo alimentados con dietas con bajo (14,6g.100g MS^{-1}) y alto (18,6g.100g MS^{-1}) contenido de CP y bajo (LWSC= 21g. 100g MS^{-1}), medio (MWSC= 24g.100g^{-1}) y alto (27g.100g MS^{-1}) contenido de WSC a 3 diferentes niveles de hidratos de carbono solubles (WSC) y 2 niveles de proteína bruta en la dieta (CP). Barras de error= 0,150, error estándar de la media. Letras diferentes indican diferencias estadísticamente significativas (P<0.05)

Table 4. Nitrogen digestion and bacterial crude protein synthesis of annual ryegrass based diets differing in water soluble carbohydrates and soluble protein content, fed to continuous culture fermenters

Tabla 4. Digestión de la fracción nitrogenada y síntesis de proteína microbiana en dietas basadas en raigrás difiriendo en contenido de hidratos de carbono soluble y proteína bruta, usando fermentadores de flujo continuo

g.100 g DM^{-1}	CP level		SEM	WSC level			SEM	*P*- value			
	LCP	HCP		LWSC	MWSC	HWSC		CP	WSC lin	WSC qua	WSC x CP
N intake (g.d^{-1})	2.38	3.03		2.69	2.69	2.74					
N outflow (g.d^{-1})	0.74	0.75	0.03	0.76	0.74	0.74	0.04	NS	NS	NS	NS
N digestion (g.100 g^{-1})	68.96	74.99	1.41	71.31	72.25	72.36	1.63	**	NS	NS	NS
Bact N.total N outflow^{-1}	0.39	0.41	0.05	0.40	0.39	0.42	0.05	NS	NS	NS	NS
CP synth (g.100 ADDM^{-1})	8.52	9.35	0.65	8.72	8.53	9.56	0.81	NS	NS	NS	NS
CP synth (g.100 TDDM^{-1})	7.42	8.03	0.50	7.59	7.44	8.15	0.61	NS	NS	NS	NS

Water soluble carbohydrates: LWSC = 21g.100g DM^{-1}, MWSC: 24g.100g DM^{-1}, HWSC 27g.100g DM^{-1}; CP (crude protein) content: LCP: 14.6g.100g DM^{-1}, HCP: 18.6g.100g DM^{-1}. N= nitrogen. CP synth = bacterial crude protein synthesis, expressed as g.100g of apparent digested DM^{-1} (ADDM) and as g.100g of truly digested DM^{-1} (TDDM). NS= not significant. Significance of the CP, WSC and their interactions were denoted by †=P<0.10, *=P<0.05, **=P<0.01

LWSC, MWSC and HWSC, nivel bajo, medio y alto de hidratos de carbono solubles, 21, 24 y 27g WSC.100g DM^{-1}. LCP y HCP, nivel bajo y alto de proteína bruta, 14,6 y 18,6g.100g MS^{-1}. N= nitrógeno, CP synth= síntesis de proteína microbiana, expresado como g.100g de MS aparentemente digerida^{-1} (ADDM) y como g.100g de MS realmente digerida^{-1} (TDDM). NS= no significativo. La significancia de CP, WSC y sus interacciones se señala por: †=P<0,10, *=P<0,05, **=P<0,01

or by grazing management strategies (Gregorini *et al*., 2006) could therefore lead to improvements in N use at rumen level, which is expected to be translated into more efficient N use for milk and beef production (Merry *et al*., 2006). In grazing systems in particular, a more efficient N use could lead to a decrease in the N urinary excretion, reducing N leaching into the soil, which has become an environmental concern in certain areas (Miller *et al*., 2001).

It has been suggested that ruminal ammonia concentrations threshold would be around 5 $mg.100mL^{-1}$ for an efficient microbial protein synthesis, with synthesis being impaired below that threshold (Satter & Slyter, 1974). Our ammonia concentration values in the low CP treatments were close to this level ($6.12mg.100mL^{-1}$). There was a positive interaction between WSC and CP levels. We believe that it is possible that at the lower level of CP and soluble protein availability, ammonia concentration became limiting for further increase in microbial protein synthesis despite greater WSC supply. This would explain the differences in our findings and those of Mansfield *et al*. (1994) or Henning *et al*. (1991), who did not find significant interactions between non-fibrous carbohydrates level and degradable protein intake. In their case, the report shows that ammonia levels in fermentation culture were not limiting even at the lowest levels of degradable protein intake, whereas in our case we were close to the threshold stated by Satter and Slyter (1974). It should also be considered that in vivo systems (i.e., live animals) have mechanisms for N recirculation through saliva (Hall & Huntington, 2008), whereas in vitro systems (i.e., continuous culture fermenters) lack this property.

Implications

It seems clear that crude protein and water soluble carbohydrate ratio in forages has an effect on microbial protein synthesis efficiency. Proper knowledge of forage composition should help to define strategies for an improvement of nitrogen use efficiency. High sugar grasses might help to improve nitrogen use, especially in the case of forages high in crude protein.

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