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In vitro evaluation of agro-industrial by-products replacing soybean meal in two different basal diets for ruminants

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Introduction

Large number of by-products from the agricultural industry can potentially be used as protein sources in diets to dairy cows. The increasing demand for alternative dietary protein supplements in ruminant production systems is due to a growing requirement for a more sustainable food production from the livestock industry. However, use of agro-industrial by-products in diets to dairy cows and beef cattle have to be efficient in terms of nutrient utilization, be complementary to basal feed ingredients and not impair production. Since *in vivo* studies are very expensive and laborious, using an *in vitro* gas production technique enables identification of by-products which can efficiently replace conventional ingredients. Recently, there has been great progress in the development of the automated gas *in vitro* technique, which enables treatment evaluation of ruminal fermentation profiles, diet digestion rates (Huhtanen *et al*., 2008), methane (CH_4) production (Ramin and Huhtanen, 2012) and estimation of utilizable crude protein (uCP; Edmunds *et al*., 2012). The aim of this study was to evaluate effects of levels of agro-industrial by-products replacing soybean meal in diets based on silage and barley or beet fibre on neutral detergent fibre (NDF) digestibility, true organic matter (OM) digestibility, uCP, fermentation parameters and CH_4 production *in vitro*.

Materials and Methods

The two basal diets used as controls for the *in vitro* incubations were grass silage:barley and grass silage:beet fibre in a ratio 600:400 g/kg of dietary dry matter (DM). Soybean meal (SBM) was used as a conventional crude protein source and was replaced by heat treated rapeseed meal (Expro®), dried distillers grain with solubles (Agrodrank™90) (DG), rapeseed cake (RSC) and rapeseed meal (RSM). Inclusions were made at two levels of crude protein (CP) concentration in the diets, differing by 2%-units. Basal, first and second levels of by-product inclusion resulted in 14.6, 16.6, and 18.6% dietary CP, respectively, for diets based on silage and barley. In diets based on silage and beet fibre, dietary CP was 12.6, 14.6 and 16.6%, respectively. All treatments had the same silage:barley or silage:beet fibre ratio across experimental diets.

Two lactating Swedish Red cows fed a diet of 600 g/kg grass silage and 400 g/kg concentrate on DM basis *ad libitum* were used for *in situ* incubation for iNDF analysis, and for collection of rumen fluid for the *in vitro* incubations (Cone *et al*., 1996). Rumen fluid was collected from the same cows for all three *in vitro* incubations. The collected rumen fluid from each cow was strained separately through a double layer of cheesecloth into pre-heated (39°C) steel thermoses that had previously been flushed with carbon dioxide and immediately taken to the laboratory. In the laboratory, rumen fluid was homogenized and filtered through four layers of cheesecloth and kept in a water bath at 39°C under CO_2 saturation. Prior to incubation, the rumen fluid was pre-incubated during 3 h with a carbohydrate mixture. In this procedure, a mixture of maltose, starch, xylose, pectin, and $NaHCO_3$ was added to the rumen fluid, which was stirred for 10 minutes. After 30 minutes, the top layer of foam was removed

with a vacuum pump and the stirrer was turned on again. The rumen fluid was then incubated at 39ºC under a constant flush of CO_2 for an additional 2.5 h. After pre-incubation, the rumen fluid was mixed with a low-N bicarbonate buffer, micro and macro minerals and resazurin.

Diets of 500 mg were previously weighed directly in 250-mL serum bottles (Schott, Mainz, Germany), which were flushed with CO_2. Diets were then incubated in 60 mL of the buffered rumen fluid for 48 h. Incubations were conducted at 39ºC and the bottles were continually agitated. All diets were incubated in 3 consecutive runs, resulting in 4 replicates per diet, including one blank per bath. Diets were randomized within baths and among baths in subsequent runs. Gas production was automatically recorded and corrected to normal atmospheric pressure (101.3 kPa; Cone *et al*., 1996). Mean blank gas production within run was subtracted from the sample gas production.

Gas samples were drawn from each bottle by a gas tight syringe (Hamilton, Bonaduz, Switzerland) at 24 and 48 h of incubation. Methane production was calculated as described by Ramin and Huhtanen (2012). Samples of 0.6 mL were taken and preserved with 0.024 mL of 18 M H_2SO_4 at 8, 16, 24, and 30 h after incubation for ammonia nitrogen (NH_3-N) analysis and estimation of uCP at 16 h as described by Edmunds *et al*. (2012):

$$\text{uCP (g/kg)} = \frac{\text{NH3N}_{\text{blank}} + \text{N}_{\text{sample}} - \text{NH3N}_{\text{sample}}}{\text{weight (mg DM)}} \text{ x } 6.25 \text{ x } 1000$$

Another sample of 0.6 mL of rumen fluid was collected at 48 h of incubation from the bottles and immediately stored at -20°C until processed for VFA determination. Discrete and total VFA production was calculated after subtracting mean blank VFA concentration from sample concentration. After 48 hours incubation, all flasks were removed from the baths and placed on ice to stop fermentation. Residues were quantitatively transferred to 11-µm bags (Saatifil PES; Saatitech S.p.A., Veniano, Como, Italy) and analysed for NDF, according to Mertens (2002). *In vitro* true OM digestibility was also determined for the diets, considering OM of individual feeds and residue after incubation.

Residual moisture of all feed samples was determined by oven drying for 16 h at 105°C. Ash concentration was determined by ignition of the dried sample at 500°C for 4 h. The indigestible NDF (iNDF) concentration was determined by a 12-d *in situ* ruminal incubation according to Krizsan *et al*. (2015). The samples were analyzed for NDF using a heat stable α-amylase (Mertens, 2002) in an ANKOM200 Fiber Analyzer (Ankom Technology Corp., Macedon, NY, USA). Values of NDF and iNDF were expressed on an ash-free basis. Concentrations of N were determined by Kjeldahl digestion of 1.0 g sample in 12 M sulfuric acid using Foss Tecator Kjeltabs Cu (Höganäs, Sweden) in a Block Digestion 28 system (SEAL Analytical Ltd., Mequon, WI, USA) with determination of total N by continuous flow analysis using an Auto Analyzer 3 (SEAL Analytical Ltd., Mequon, WI, USA). Individual VFA concentrations in rumen fluid samples were determined using a Waters Alliance 2795 HPLC system with Waters 2414 RI detector (Waters Corporation, Milford, MA, USA) as described by Ericson and André (2010), and NH_3, according to the method provided by the SEAL Analytical (Method nr G-102-93 multitest MT7) using the AutoAnalyzer 3.

The data was analysed using the GLM procedure (SAS Inc. 2002-2003, Release 9.2; SAS Inst., Inc., Cary, NC) of SAS at 5% of probability. The sum of squares was further partitioned into orthogonal polynomial contrasts, where SBM was contrasted against by-products, and linear and quadratic responses to level of by-products.

Results and Discussion

The chemical composition of the silage, barley, beet fibre, soybean meal, and by-products are in Table 1. Levels of CP in the by-products ranged between 315 and 392 g/kg DM, while in the soybean meal, it was 496 g/kg DM.

Table 1 Chemical composition of silage, barley, beet fibre, soybean meal and by-products (g/kg DM unless otherwise stated)

					By-products			
Item	Silage	Barley	Beet fibre	SBM	Expro	DG	RSC	RSM
DM, g/kg	255	953	917	854	906	877	921	911
OM	842	926	848	925	837	827	859	840
CP	157	129	78	496	387	315	378	392
NDF	611	239	339	237	322	288	251	270
NSC	286	714	578	688	584	589	670	641
iNDF	102	42	30	6	129	63	109	118

DM: dry matter; OM: organic matter; CP: crude protein; NDF: neutral detergent fibre; NSC: non-structural carbohydrate; iNDF: indigestible neutral detergent fibre; SBM: soybean meal; Expro: heat treated RSM, DG: distillers grain; RSC: rapeseed cake; RSM: rapeseed meal.

In Table 2, results indicate that Expro, RSC and RSM decreased ($P<0.05$) NDF and true OM digestibility when replacing soybean meal in diets based on silage and barley. However digestibilities were not affected ($P>0.05$) by DG inclusion, which may be explained by its low proportion of iNDF, providing more digestible matter compared to the other by-products. Utilizable CP increased ($P<0.05$) for all by-products replacing soybean meal. A high uCP level, defined as the sum of microbial crude protein (MCP) and rumen undegraded protein (RUP) (Edmunds *et al*., 2012), indicates a higher proportion of utilisable protein substrate available in the duodenum. In the *in vitro* uCP estimation, RUP and MCP are simultaneously estimated and cannot be differentiated. According to Edmunds *et al*. (2012), validation using *in vivo* data is recommended. Even though it was not possible to differentiate the two sources, it is likely that Expro, RSC and RSM have a relatively high proportion of RUP as NDF and true OM digestibilities decreased ($P<0.05$) when those by-products replaced soybean meal.

Distillers grain replacing soybean meal in diets based on silage and barley decreased ($P<0.05$) acetate and increased ($P<0.05$) propionate proportions (Table 2), while they were not affected ($P>0.05$) by the other by-products. Furthermore, when soybean meal was replaced by DG or Expro, CH_4 production decreased ($P<0.05$). None of the by-products affected ($P>0.05$) total VFA and digestion rate in these diets.

Incremental levels of by-products in the diets based on silage and barley linearly increased ($P>0.05$) (Table 2) true OM digestibility, uCP, isobutyrate and valerate, and linearly decreased ($P<0.05$) acetate and butyrate proportions. The increase in dietary crude protein concentration increased ($P<0.05$) uCP, which suggests that all by-products tested are potential protein feed sources without detrimental effect on uCP. Total VFA, NDF digestibility, digestion rate and CH_4 production were not affected ($P>0.05$) by the inclusion level of by-product.

Table 2 Effect of increasing level of agro-industrial by-products replacing soybean meal on digestibility, estimated utilizable crude protein, fermentation parameters and methane production in diets based on silage and barley

	Basal 14.6%	Diets 16.6% CP					Diets 18.6% CP						P-value[a]				
Item	CP	SBM	Expro	DG	RSC	RSM	SBM	Expro	DG	RSC	RSM	SEM	C1	C2	C3	C4	Lin
NDFD, g/kg	781	790	776	804	776	789	811	769	784	769	769	7.2	<0.01	0.37	<0.01	<0.01	0.68
TOMD, g/kg	849	856	849	864	852	857	869	849	859	855	853	3.3	<0.01	0.60	0.01	0.02	0.03
uCP, g/kg DM	150	158	164	165	161	163	167	179	182	172	171	0.8	<0.01	<0.01	<0.01	<0.01	<0.01
Total VFA, mmol/l	91.9	91.9	87.6	90.8	92.6	93.2	92.5	92.5	90.0	87.4	88.6	1.96	0.27	0.36	0.26	0.51	0.37
Molar proportions, mmol/mol																	
Acetate	591	590	586	585	594	591	589	587	579	584	590	1.9	0.15	<0.01	0.89	0.66	0.01
Propionate	228	226	229	233	221	228	227	227	241	227	224	1.9	0.47	<0.01	0.18	0.71	0.41
Butyrate	111	111	107	105	110	109	108	109	102	107	108	1.1	0.20	<0.01	0.42	0.30	<0.01
Isobutyrate	19	20	20	19	20	19	20	20	20	21	20	0.4	0.94	0.12	0.33	0.91	<0.01
Valerate	27	27	29	31	29	28	28	31	31	32	30	0.5	<0.01	<0.01	<0.01	0.03	<0.01
Isovalerate	25	26	28	27	26	25	27	26	27	29	28	1.1	0.60	0.90	0.50	0.91	0.10
k_d, 1/h	0.073	0.073	0.072	0.074	0.077	0.077	0.077	0.068	0.077	0.077	0.082	0.0028	0.09	0.80	0.42	0.10	0.26
CH_4, ml/g DM	53.5	53.3	52.1	50.9	49.6	51.8	53.9	51.4	49.8	51.9	52.9	1.06	0.09	<0.01	0.01	0.24	0.33

CP = crude protein; SBM = soybean meal; DG = distillers grain; RSC = rapeseed cake; RSM = rapeseed meal; SEM = standard error of mean; NDFD = neutral detergent fibre digestibility; TOMD = true organic matter digestibility; uCP = utilizable crude protein; Total VFA = volatile fatty acids (sum of all individual acids); kd = diet digestion rate. [a]C1 = SBM vs. Expro; C2 = SBM vs. DG; C3 = SBM vs. RSC; C4 = SBM vs. RSM; Lin = linear effect of supplementary inclusion level; Quad = quadratic effect of supplementary inclusion level.

Table 3 Effect of increasing level of agro-industrial by-products replacing soybean meal on digestibility, estimated utilizable crude protein, fermentation parameters and methane production in diets based on silage and beet fibre

	Basal 12.6%	Diets 14.6% CP					Diets 16.6% CP						P-value[a]				
Item	CP	SBM	Expro	DG	RSC	RSM	SBM	Expro	DG	RSC	RSM	SEM	C1	C2	C3	C4	Lin
NDFD, g/kg	799	819	794	830	791	795	822	801	798	793	790	11.7	0.05	0.56	0.01	0.02	0.99
TOMD, g/kg	841	850	837	856	838	839	855	845	846	846	842	6.0	0.05	0.85	0.09	0.05	0.33
uCP, g/kg DM	140	151	152	155	152	151	158	167	171	162	162	1.0	<0.01	<0.01	<0.01	0.08	<0.01
Total VFA, mmol/l	90.1	94.9	87.8	91.1	95.4	90.3	97.7	94.4	92.4	92.8	95.4	2.66	0.06	0.09	0.41	0.20	0.08
Molar proportions, mmol/mol																	
Acetate	633	628	622	623	627	623	623	619	613	620	622	2.6	0.07	<0.01	0.45	0.28	<0.01
Propionate	230	222	228	231	222	228	224	225	238	228	226	1.6	0.03	<0.01	0.17	0.02	0.70
Butyrate	81	85	84	80	85	83	86	86	81	83	86	1.3	0.50	<0.01	0.20	0.34	0.05
Isobutyrate	17	18	19	18	18	18	18	19	17	18	18	0.7	0.24	0.28	0.86	0.70	0.32
Valerate	22	24	26	26	25	24	25	28	27	26	26	0.6	<0.01	0.01	0.30	0.54	<0.01
Isovalerate	24	23	26	23	23	25	24	23	24	25	22	1.1	0.30	0.94	0.58	0.95	0.73
k_d, 1/h	0.076	0.077	0.074	0.081	0.079	0.077	0.078	0.077	0.082	0.088	0.083	0.0030	0.54	0.24	0.06	0.43	0.05
CH_4, ml/g DM	52.8	52.1	52.0	50.9	49.8	51.9	55.6	51.6	51.8	54.9	51.1	1.10	0.04	0.01	0.14	0.02	0.36

CP = crude protein; SBM = soybean meal; DG = distillers grain; RSC = rapeseed cake; RSM = rapeseed meal; SEM = standard error of mean; NDFD = neutral detergent fibre digestibility; TOMD = true organic matter digestibility; uCP = utilizable crude protein; Total VFA = volatile fatty acids (sum of all individual acids); kd = diet digestion rate. [a]C1 = SBM vs. Expro; C2 = SBM vs. DG; C3 = SBM vs. RSC; C4 = SBM vs. RSM; Lin = linear effect of supplementary inclusion level; Quad = quadratic effect of supplementary inclusion level.

For diets based on silage and beet fibre (Table 3), replacement of soybean meal by RSC or RSM decreased (P<0.05) NDF digestibility, while true OM digestibility was not affected (P>0.05). None of the digestibilities were affected (P>0.05) by Expro or DG replacing soybean meal. Similar to the diets based on silage and barley, diets based on silage and beet fibre, uCP increased (P<0.05) for all by-products replacing soybean meal, except for RSM, where there was only a tendency (P<0.09). This indicates that the by-products used in this *in vitro* experiment are good feed protein sources. However, intestinal digestibility of uCP of the different diets can vary and data on that is needed in order to fully evaluate diet protein values.

Distillers grain decreased (P<0.05) (Table 3) acetate and butyrate, and increased (P<0.05) propionate when replacing soybean meal in diets based on silage and beet fibre. Moreover, propionate also increased (P<0.05) when Expro or RSM replaced soybean meal. Except for RSC, increasing level of by-product decreased (P<0.05) CH_4 production. A reason for this could be that propionate and CH_4 production requires H_2, and since propionate production increased, less hydrogen was available for CH_4 production. The high protein concentration of the by-products could also act in formation of bicarbonate from CO_2 and, thereby, reducing CO_2 production (Cieslak *et al*., 2013). According to Menke *et al*. (1979) and Ramin and Huhtanen (2013) there is a high correlation between CH_4 production and digestibility. This was obvious in the RSM diet, where NDF digestibility decreased (P<0.05), true OM digestibility tended to decrease (P<0.06) and also CH_4 production decreased (P<0.05). The same pattern was also observed when soybean meal was replaced by RSC in diets based on silage and barley, in accordance with Jentsch *et al*. (2007). There is a negative correlation between uCP and gas production (Vaga *et al*., 2016), which was also seen in this study when Expro, DG and RSM replaced soybean meal in diets based on silage and beet fibre. Moreover, the same pattern was evident for diets based on silage and barley, with DG and RSC replacing soybean meal. None of the by-products replacing soybean meal affected (P>0.05) total VFA and digestion rate in diets based on silage and beet fibre.

There was a positive linear effect (P<0.05) (Table 3) of by-product level on uCP and valerate, and a negative linear effect (P<0.05) for acetate in diets based on silage and beet fibre. The increase in uCP concentrations was expected due to its correlation with dietary CP. There was a quadratic effect (P<0.05) of supplementary inclusion level of by-product on propionate and CH_4 production, where the first inclusion level decreased, followed by an increase for the highest level for both parameters.

In general, detrimental effects (e.g., digestibility) were more prominent in the diets based on silage and barley than in the diets based on silage and beet fibre. It may have been due to its lower CP and higher NDF concentration (Table 1) in comparison with barley.

Conclusions

Caution should be taken when by-products replace soybean meal in diets based on silage and barley with respect to detrimental effects on digestibility in spite of an increase in uCP. However, this does not seem to be a problem in a diet based on silage and beet fibre, with a potential benefit in the form of reduced methane production increased uCP production. It should be noted that intestinal digestibility of uCP is not known and requires evaluation in order to assess the true protein value of the diet.

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