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Effects of ricinoleic acid from castor oil and cashew nutshell liquid on nutrient digestibility and ruminal fermentation in dairy heifers

Efeitos do ácido ricinoleico do óleo de mamona e do líquido da castanha de caju sobre a digestibilidade de nutrientes e fermentação ruminal de novilhas leiteiras

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ABSTRACT

This study aimed to evaluate the effects of combining functional oils (FO) [ricinoleic acid (RA) and cashew nutshell liquid (CNSL)] on nutrient intake and total-tract apparent digestibility, ruminal fermentation, nitrogen utilization, and predicted rumen microbial protein (Pmic) in heifers. Twelve Jersey heifers (14±0.6 months and 264±18.7 kg BW) were assigned to a 4 × 4

ISSN 1519 9940

Latin square experiment with the following treatments: Control (CON), diet without feed additives; Ricinoleic acid, dietary inclusion of RA at 2 g kg^{-1} dry matter (DM); Cashew nutshell liquid, dietary inclusion of CNSL at 2 g kg^{-1} DM; and a mixture of 1 g kg^{-1} DM of RA and 1 g kg^{-1} DM of CNSL (RA+CNSL). Heifers were allowed 14 d for treatment adaptation followed by 5 days of sampling. Total feces collection was performed to determine digestibility. Rumen fluid was collected to determine short-chain fatty acids (SCFA) concentration. Urine samples were collected for nitrogen and purine derivatives analyses. Feeding RA decreased intake of DM, but increased crude protein (CP) digestibility and ruminal acetate concentration. Feeding CSNL increased NDF digestibility and lowered Pmic. The association of RA+CNSL increased neutral detergent fiber (NDF) digestibility and ruminal concentration of total SCFA without affecting DM intake. Feeding RA treatment decreased N intake and N excreted in feces and urine. CNSL group had the highest values of N balance. Heifers fed RA had lower Pmic than CNSL and RA+CNSL. The association of RA+CNSL improved digestibility of fiber and increased ruminal concentration of SCFA without altering N balance and Pmic.

Key words: *Anacardium occidentale*, feed additive, *Ricinus communis* L, rumen modulator

RESUMO

O objetivo deste estudo foi avaliar os efeitos da combinação de óleos funcionais (OF) [ácido ricinoleico (AR) e líquido da castanha de caju (LCC)] no consumo e digestibilidade de nutrientes, fermentação ruminal, utilização de nitrogênio e estimativa de proteína microbiana de novilhas leiteiras. Doze novilhas da raça Jersey (14±0,6 meses e 264±18,7 kg PV) foram alocadas em um experimento de quadrado latino 4×4 contendo os tratamentos a seguir: controle (CON), uma dieta sem aditivos; Ácido ricinoleico, inclusão de 2g kg^{-1} de matéria seca (MS) de AR na dieta; Líquido da castanha de caju (LCC), inclusão de 2 g kg^{-1} MS de LCC na dieta; e uma mistura de 1 g kg^{-1} MS de AR e 1 g kg^{-1} MS de LCC na dieta (AR+LCC). Cada período experimental tinha 14 dias para adaptação aos tratamentos e 5 dias de coleta. O consumo de alimentos foi mensurado diariamente, e uma coleta total de fezes foi realizada durante 3 dias consecutivos de cada período experimental para determinar a digestibilidade de nutrientes. Amostras de fluído ruminal foram coletadas no último dia de cada período para determinação da concentração de ácidos graxos de cadeia curta. Amostras de urina foram coletadas no dia 18 de cada período para análises de nitrogênio e derivados de purina. O fornecimento de AR diminuiu o consumo de MS e proteína bruta (PB), mas aumentou a digestibilidade de proteína bruta e concentração ruminal de acetato. O fornecimento de LCC não afetou o consumo, mas aumentou a digestibilidade da fibra em detergente neutro (FDN). A associação de AR+LCC aumentou a digestibilidade da PB e FDN como também a concentração total de ácidos graxos de cadeia curta no rúmen, sem alterar o consumo de MS. O tratamento AR diminuiu a ingestão de N como também a excreção de N pelas fezes e urina. As novilhas do grupo LCC tiveram os maiores valores de N absorvido e retido enquanto as novilhas do grupo AR tiveram os menores valores. As novilhas do grupo RA tiveram

ISSN 1519 9940

menor estimativa de proteína microbiana em comparação com aquelas no grupo LCC e AR+LCC mas não diferiram do CON. Em conclusão, a associação de AR+LCC melhorou a digestibilidade da fibra e aumentou a concentração do total de ácidos graxos de cadeia curta no rúmen sem alterar o balanço de N e estimativa da proteína microbiana no rúmen.

Palavras-chave: *Anacardium occidentale*, aditivo alimentar, *Ricinus communis* L, modulador ruminal

INTRODUCTION

Functional oils (FO), such as cashew nutshell liquid (CNSL) and ricinoleic acid (RA) from castor oil, are chemical substances extracted from *Anacardium occidentale* seeds husk and *Ricinus communis* L. seeds by distillation, compression and solvents that present health benefits besides their nutritive properties (Ferreira de Jesus et al., 2016). Ricinoleic acid is similar to oleic acid, the only difference is a hydroxyl group present in RA. For this reason, RA is also called hydroxyoleic acid (Alves et al., 2017). Although the castor bean toxicity is described since the ancient times, the oil extracted from the castor bean is not toxic as the protein responsible for its toxicity (ricin) is not lipid soluble. Thus, the toxic component is restricted only to the whole castor bean. Ricinoleic acid is known by its antimicrobial properties (Novak et al., 1961); thus, RA has been studied as a possible modulator of ruminal fermentation to be used as an alternative to ionophores to improve growth, feed intake, and efficiency.

Cashew nutshell liquid is a by-product of cashew nut processing and has a variety of industrial uses (Menon et al., 1985; Lubi and Thachil, 2000). Natural CNSL contains mainly 4 constituents, cardanol, cardol, anacardic acid, and 6-methyl cardol (1.2, 11.3, 64.9, and 2.0% by weight, respectively), which are mixtures of constituents differing in side-chain unsaturation (Lubi & Thachil, 2000). The antibacterial properties of anacardic acid are well known and may be species-specific (Van Nevel et al., 1971, Kubo et al., 1993). These FO have a high potential for modulating ruminal fermentation mainly due to their antimicrobial properties (Ferreira et al., 2016). These co-products have significant efficacy when fed to beef cattle (Gandra et al., 2012) and dairy cows (Gandra et al., 2014; Branco et al., 2015), but literature lack information on the efficacy of feeding the combination of RA and CNSL. We hypothesized that RA and CNSL dietary combination improves the nutrient utilization in dairy heifers. The objective of this study was to evaluate the effects of combining two FO (RA and CNSL) on intake and total-tract apparent digestibility of nutrients, ruminal fermentation, nitrogen utilization, and predicted rumen microbial protein in dairy heifers.

MATERIALS AND METHODS

This experiment was carried out under the approval of Bioethics Committee (protocol #044/2017) from the Federal University of Grande Dourados (UFGD), Dourados, MS,

ISSN 1519 9940

Brazil. Twelve Jersey heifers (14 ± 0.6 months of age and 264 ± 18.7 kg BW) were assigned to a 4 × 4 Latin square design with the following treatments: Control (CON), diet without feed additives; Ricinoleic acid, dietary inclusion of RA at 2 g kg^{-1} diet dry matter (DM); Cashew nutshell liquid (CNSL), dietary inclusion of CNSL at 2 g kg^{-1} DM; and a mixture of 1 g kg^{-1} DM of RA and 1 g kg^{-1} DM of CNSL (RA+CNSL). Treatments were top-dressed and fed in equal amounts at each feeding. Cashew nutshell liquid used in this study was the same described by Branco et al. (2015) and contained: cardanol (73.3%), cardol (16.4%), and 2-methylcardol (3.0%). Ricinoleic acid was offered as a dry white powder and dosage was based on results of Gandra et al. (2014). Experimental periods consisted of 14 days for treatment adaptation followed by 5 days of sampling and data collection. Diets were formulated according to NRC (2001) to be isonitrogenous and targeting 600 g d^{-1} average daily gain (Table 1). Heifers were housed in a barn with individual pens (12 m^2 area) equipped with individual waterers and feed bunks. Diets were provided twice a day (0600 and 1300 h) as total mixed ration targeting refusals at 5-10% as-fed.

Table 1 - Ingredients and chemical composition of the experimental diet (g kg^{-1}, unless stated).

Item	Diet
Ingredient	
Corn silage	600
Ground corn	210
Whole raw soybean	154
Urea	19.5
Mineral mix[1]	19.5
Chemical	
Dry matter, g kg^{-1} as-fed	522
Organic matter	921
Crude protein	158
Ether extract	55.5
aNDF	385
Acid detergent fiber	237
Non-fiber carbohydrate	367
Total digestible nutrient[2]	710
Net energy[2], Mcal kg^{-1}	1.62

[1]Contained per kilogram: 134 g Ca, 60 g P, 10 g Mg, 110 g Na 12 g S, 30 mg Se, 60 mg I, 150 mg Co, 6,000 mg Zn, 2,500 mg Fe, and 4,500 mg Mn.
[2]Calculated according to NRC (2001).

ISSN 1519 9940

Samples of feeds and refusals from each heifer were collected daily during the sampling period and pooled per period for further analyses. Samples were analyzed for DM (method 950.15), ash (method 942.05), OM (DM − ash), crude protein (N × 6.25; method 984.13), and ether extract (method 920.39) according to AOAC (2000). Samples were also assessed for neutral detergent fiber (aNDF) and acid detergent fiber (ADF) according to Van Soest et al. (1991). Dietary contents of total digestible nutrients and net energy were calculated according to NRC (2001). Total feces sampling was carried out on days 15, 16, and 17 of each period and a representative sample (10% on wet basis) from each cow was frozen until chemical analysis, as described earlier. Total-tract digestibility was calculated as the total nutrient intake minus the nutrient excreted in feces, divided by nutrient intake.

Ruminal fluid samples were collected on the last day of each period, 4 h after the morning feeding using an esophageal gavage and attached to a vacuum pump. The first 250 mL of fluid was discarded to avoid saliva contamination, then pH was measured (pH 1500, Instrutherm, Sao Paulo, Brazil). Rumen fluid (1,600 µL) was mixed with methanoic acid (400 µL), centrifuged (7,000 × *g* for 15 min at 4 °C), and the supernatant harvested for short-chain fatty acids analysis. Rumen NH_3-N concentration was determined by the colorimetric phenol-hypochlorite method (Broderick and Kang, 1980). Ruminal concentrations of short-chain fatty acids (SCFA) were determined through gas chromatography (modelGC-2104, Shimadzu, Tokyo, Japan) according to Erwin et al. (1961). The gas chromatograph was equipped with a split injector and dual flame ionization detector with the temperature set at 250 °C and capillary column (Stabilwax, Restek, Bellefonte, PA) temperature set at 145 °C. The gases used in the SCFA analyses were helium as the carrier gas (flowing at 8.01 mL min^{-1}), hydrogen as the fuel gas (pressure of 60 kPa), and synthetic air as the oxidizer gas (pressure of 40 kPa). An external standard was prepared with acetic, propionic, and butyric acids (Chem Service, Inc., West Chester, PA). The software GC Solution (Shimadzu) was used to calculate samples SCFA concentration. Methane production (CH_4) was calculated according to Moss et al. (2000).

Urine samples were collected on d 18 of each experimental period, 4 h after the morning feeding. Samples were filtered and stored frozen for determination of urine N content according to AOAC (2000). Daily urinary volume was estimated as the ratio between total creatinine excretion and creatinine concentration in urine samples (Oliveira et al., 2001). Urinary creatinine excretion (CE) was calculated as: CE (mg kg^{-1} BW) = 32.27−0.01093 × BW (kg) (Chizotti et al., 2008). Urine creatinine concentration was determined by an enzymatic colorimetric method using commercial kits (Laborlab, Osasco, Brazil) and absorbance was measured on biochemistry analyzer (SBA-200, CELM, Sao Caetano do Sul, Brazil). Microbial protein synthesis was estimated as the sum urinary excretion of allantoin and uric acid [purine derivatives (PD)] according to Chen and Gomes (1992). Ten mL of urine was

diluted in sulfuric acid solution (40 mL at 0.036 *N*) to avoid PD destruction and uric acid precipitation. Allantoin and uric acid concentrations in urine were determined by a colorimetric method according to Fujihara et al. (1987). Absorbed microbial purines (Pabs, mmol d^{-1}) were calculated as follows: $Pabs = (PD - 0.512 \times BW^{0.75}) \div 0.70$, where 0.70 is the recovery of absorbed purines and $0.512 \times BW^{0.75}$ is the endogenous excretion of PD (Gonzalez-Ronquillo et al., 2003). Predicted microbial N (Nmic, g d^{-1}) was calculated according to Chen and Gomes (1992): $Nmic = (70 \times Pabs) \div (0.83 \times 0.134 \times 1000)$, where 70 is the N content in purines (mg mol^{-1}), 0.134 is the ratio of purine N to bacterial N (Valadares et al., 1999), and 0.83 is the intestinal digestibility of microbial purines. Heifers were weighed in a scale for large animals on d 19 of each period before the morning feeding.

Data were submitted to analysis of variance using the PROC MIXED of SAS (version 9.4, SAS Institute, Cary, NC), according to the following model:

$$Y_{ijkl} = \mu + T_i + P_j + S_k + a_l + e_{ijkl}$$

where: Y_{ijkl} is the dependent variable, μ is the overall mean; T_i is the fixed effect of treatment (i = 1 to 4); P_j is the fixed effect of period (j = 1 to 4); S_k = is the fixed effect of Latin Square (k = 1 to 3); a_l is the random effect of animal within square (l = 1 to 12); and e_{ijkl} is the random experimental error. Treatment differences were evaluated through adjusted Tukey's test and degrees of freedom corrected by the Kenward and Rogers option. Significance level was set at 0.05 and tendencies considered when $0.05 < P < 0.10$.

RESULTS AND DISCUSSION

We hypothesized that either RA and CSNL would improve nutrient digestibility and rumen fermentation, whereas their combination would have a positive synergistic impact on these variables. This experiment did show positive synergistic effect on NDF digestibility and ruminal concentrations of propionate and total short-chain fatty acids, whereas both FO modulated nutrient utilization and rumen fermentation at some extent. Feeding RA to dairy heifers decreased ($P \leq 0.044$) intake of DM, organic matter, and crude protein (Table 2).

Table 2 - Intake and total-tract apparent digestibility of nutrients, and rumen fermentation of dairy heifers fed functional oils.

	Treatment[1]				SEM	*P*-value
Item	COM	RA	CNSL	RA+CNSL		
Intake, kg d^{-1}						
Dry matter	13.0[a]	11.6[b]	13.8[a]	12.5[ab]	0.53	0.044
Organic matter	11.1[a]	9.96[b]	11.9[a]	10.4[ab]	0.56	0.028
Crude protein	2.10[a]	1.87[b]	2.22[a]	2.02[ab]	0.08	0.018
aNDF	4.43	3.98	4.70	4.13	0.28	0.223
Digestibility, g kg^{-1}						
Dry matter	737	760	775	769	1.27	0.485

ISSN 1519 9940

Organic matter	751	769	784	788	1.52	0.245
Crude protein	802^{b}	832^{a}	829^{a}	841^{a}	1.19	0.033
aNDF	678^{b}	671^{b}	702^{a}	731^{a}	2.31	0.031
Rumen fermentation						
pH	6.45	6.39	6.41	6.48	0.02	0.601
NH-N_3, mg dL^{-1}	18.6	17.8	18.2	19.2	0.21	0.703
Acetate, mmol L^{-1}	50.7^{ab}	54.1^{a}	49.1^{b}	52.0^{a}	2.19	0.005
Propionate, mmol L^{-1}	16.5^{b}	17.1^{ab}	16.5^{b}	20.1^{a}	1.12	0.004
Butyrate, mmol L^{-1}	10.4	8.68	8.65	9.23	0.56	0.245
Total, mmol L^{-1}	77.6^{b}	79.8^{ab}	74.2^{b}	81.3^{a}	3.17	0.002
Acetate to propionate ratio	3.17	3.24	3.09	2.96	0.14	0.457
Methane[2] mmol L^{-1}	22.4	23.1	21.0	21.6	0.98	0.088

[1]Control (CON), diet without feed additives; Ricinoleic acid (RA), dietary inclusion of RA at 2 g kg^{-1} DM; Cashew nutshell liquid (CNSL), dietary inclusion of CNSL at 2 g kg^{-1} DM; and a mixture of 1 g kg^{-1} DM of RA and 1 g kg^{-1} DM of CNSL (RA+CNSL).
[2]Methane = (acetic acid × 0.45) − (propionic acid × 0.275) + (butyric acid × 0.40) all variables expressed as mmol/L according to Moss et al. (2000)

Treatments with FO (RA, CNSL, and RA+CNSL) increased (P = 0.033) crude protein digestibility. Although CNSL and RA+CNSL treatments had no effect on nutrient intake, they increased (P =0.031) NDF digestibility in heifers. Agreeing with the current study, authors have reported a decrease in DM intake when feeding RA to lactating cows (Gandra et al., 2014); these authors suggested that the depressed feed intake is related to RA effect on rumen modulation for greater production of propionate (Gandra et al., 2014). Studies have shown that rumen propionate can stimulate satiety in ruminants and laboratory species (Oba and Allen, 2003; Allen, 2020). Indeed, heifers fed RA (RA or RA+CNSL) exhibited the highest ruminal concentrations of propionate and total SCFA compared with other groups.

Heifers fed RA or RA+CNSL exhibited the highest ruminal concentration of acetate, whereas those fed CON showed intermediate values, and CNSL the lowest values. The combination of RA+CNSL increased ($P \leq 0.004$) ruminal concentrations of propionate and total SCFA in comparison with CON and CNSL but did not differ from RA group. In this study, no positive synergistic effects between RA and CSNL were detected for nutrient intake, but the combination of these two FO improved NDF digestibility and ruminal concentration of total SCFA and propionate. An *in vitro* study also reported greater propionate but lower butyrate concentration in rumen digesta incubated with RA after 24 h (Morales et al., 2012). Furthermore, Gandra et al. (2017) found that RA can affect glucose and insulin dynamics in horses which can also be associated with the negative effects of RA on feed intake. More recently, authors did not detect differences in DM intake of low producing dairy cows fed RA at 2 g d^{-1} (Pawar et al., 2021). The antimicrobial activity of RA in rumen digesta has been described elsewhere (Wallace et al.,

2007; Morales et al., 2012). Ricinoleic acid inhibited growth of bihoydrogenating bacteria (Wallace et al., 2007) and *Butyrivibrio proeoclasticus* (Morales et al., 2012). Despite the specific inhibition of rumen bacteria, the current study does not provide evidence for shifts in rumen fermentation, as RA increased both acetate and propionate concentrations in rumen fluid. The greater concentration of short-chain fatty acids may suggest an increase in rumen degradation rate of nutrients of heifers fed treatments containing RA; however, it is noteworthy that rumen concentration of SCFA is dependent on ruminal digesta liquid amount and does not always reflect an increase in microbial activity and improved fermentation (Hall et al., 2015).

Heifers fed RA treatment had lower (P = 0.018) N intake in comparison with CON and CNSL, whereas the combination of RA+CNSL had similar N intake than other treatments (Table 3). Feeding RA treatment also decreased ($P \leq 0.047$) N excretion through feces and urine in comparison with other treatments. Heifers fed the CNSL treatment had the highest ($P \leq 0.041$) values of absorbed and retained N, whereas heifers fed RA treatment exhibited the lowest values, and intermediate values were observed for heifers in CON and RA+CNSL groups . As a consequence of lower N intake, heifers fed RA had decreased N absorption and accretion. Thus, the supplementation with RA to dairy heifers should be carefully analyzed since it impaired the absorption of N-components required for protein synthesis and growth. Heifers fed CNSL diets had the greatest values for N absorption (intake of N – N excretion in feces) and N retained (N intake – N in feces – N in urine) due to the greatest CP intake and digestibility values when compared to other treatments. Cows fed treatments with RA exhibited the lowest values for excretion of uric acid, total purines, and predicted microbial protein, whereas CON had intermediate values, and CNSL had the highest values. Studies evaluating the effects of RA supplementation on rumen microbial protein synthesis lack in the literature.

Table 3 - Nitrogen utilization and rumen microbial protein synthesis of dairy heifers fed functional oils.

Item	Treatment[1]				SEM	*P*-value
	COM	RA	CNSL	RA+CNSL		
N utilization, g d^{-1}						
Nitrogen intake	336[a]	301[b]	357[a]	325[a]	5.00	0.018
Fecal N	63.9[a]	49.1[b]	53.5[a]	50.9[a]	4.04	0.024
Urinary N	24.8[a]	13.2[b]	21.3[a]	18.3[a]	6.07	0.047
Nitrogen balance[2], g d^{-1}						
Absorbed	272[ab]	251[b]	303[a]	274[ab]	6.80	0.038
Retained	247[ab]	238[b]	282[a]	255[ab]	4.70	0.041
Purine derivatives, mmol d^{-1}						
Allantoin	62.0	61.0	56.9	57.5	3.83	0.892

ISSN 1519 9940

Uric acid	19.5	15.2	18.4	12.8	1.68	0.002
Total purines	81.4^{ab}	76.2^{b}	84.3^{a}	70.3^{b}	5.10	0.024
Absorbed purines	82.8^{ab}	76.7^{b}	82.4^{a}	69.6^{b}	6.08	0.035
Predicted microbial protein, g/d	358^{ab}	348^{b}	363^{a}	316^{b}	8.60	0.038

[1]Control (CON), diet without feed additives; Ricinoleic acid (RA), dietary inclusion of RA at 2 g kg^{-1} DM; Cashew nutshell liquid (CNSL), dietary inclusion of CNSL at 2 g kg^{-1} DM; and a mixture of 1 g kg^{-1} DM of RA and 1 g kg^{-1} DM of CNSL (RA+CNSL).
[2]Absorbed N = N intake – fecal N, and Retained N = N intake – fecal N – urinary N.

Feeding CNSL to heifers did not affect intake of nutrients but increased NDF digestibility. Agreeing with the current study, Branco et al. (2015) reported no differences in nutrient intake and a tendency for increased NDF digestibility in high producing cows. Coutinho et al. (2014), however, did not detect differences in nutrient intake and digestibility when feeding CNSL to low producing dairy cows. The reasons for greater NDF digestibility are not clear since rumen NDF degradation seems to not be improved when feeding CNSL as it showed the lowest rumen concentration of acetate (the main SCFA derived from fiber degradation). Despite the primary compounds in CNSL (cardol and cardanol) have antibacterial, antiprotozoal, and antifungal properties (Stasiuk and Kozubek, 2010), feeding CNSL increased predicted rumen microbial protein in the current study. Authors, however, did not detect differences in predicted microbial protein when feeding CNSL to lactating cows (Branco et al., 2015).

Cashew nutshell liquid has been associated with increased rumen propionate production/proportion and decreased methane production in *in vitro* and *in vivo* studies with a series of substrates (Oh et al., 2017; Kang et al., 2018). This study did detect a trend towards lower methane estimate production for heifers fed CNSL when compared with other treatments. Cashew nutshell liquid has been shown to have a surfactant activity on the cell surface of two species of hydrogen and formate producing bacteria (substrates for methane production), and propionate producing species were not altered after exposure to CNSL (OH et al., 2017). These observations were associated with the presence by an outer membrane that protects cells from surfactant action in the propionate producing bacteria (*Streptococcus ruminantium* and *Megasphaera elsdenii*) while the hydrogen and formate producing bacteria lack such a membrane (*Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens*) (Chen and Wolin, 1979; Callaway et al., 1999).

Although the positive impacts on nutrient utilization and animal performance when feeding the combination of RA and CNSL have been extensively described in ovine, steers, and lactating cows (Ferreira de Jesus et al., 2016; Ghizzi et al., 2018; Michailoff et al., 2020; Serrano et al., 2020), literature lack information regarding this combination in diets of dairy heifers. To the best of our knowledge, this is the first experiment that described the positive effects of combining RA and CNSL on fiber digestibility and rumen fermentation of dairy heifers.

CONCLUSION

The combination of RA and CNSL showed positive synergistic effects on CP and NDF digestibility, as well as on ruminal concentrations of propionate and total short-chain fatty acids. Feeding RA decreased the N absorbed and retained by heifers. The combination of RA and CNSL had no effect on microbial protein in comparison with control group. Further studies with growing heifers to evaluate the effects of functional oils on performance and feed efficiency should be carried out.

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