Full-fat corn germ in diets for dairy cows as an alternative to ground corn

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SUPPLEMENTARY FILE

Detailed Methodology of Research

Two trials were conducted for the development of this work. The first (Test I) evaluated the performance of Holstein cows in the field submitted to experimental diets with five levels of replacement of GC for FFCG. The second (Test II) was an in vitro gas production experiment in a fully automated system. For the *in vitro* gas production assay, three incubations were performed in order to evaluate and predict the in vivo methane production from the experimental diets used in Test I. The tests are described separately in this section.

Test I.

Animal care and experiment location

The procedures with the animals were carried out in accordance with the guidelines of the Ethics Committee on the Use of Animals (ECUA) of the Universidade Federal Rural de Pernambuco (License nº 143/2019).

23 The experiment was carried out at the Experimental Station of the Instituto Agronômico de
24 Pernambuco (IPA), located in the municipality of São Bento do Una-PE, whose climate is
25 classified as hot semi-arid (BWh), according to the Koppen classification system (Köppen, 1948),
26 situated at latitude 08°31'22” S and longitude 36°06'40” W, with an average annual rainfall of 655
27 mm and an average temperature of 23.8°C (Farias *et al.,* 2000).
28
29 *Animals, experimental design and dietary treatments*
30 Ten multiparous Holstein cows with 90 ± 10 days in milk and yielding 24.2 ± 3.5 (mean ± SD) kg
31 of milk/d were used in the study. The cows were housed in individual pens of 24 m^2 equipped with
32 feed bins and water troughs.
33 Before the experimental period, all animals were adapted to facilities and management practices
34 for three weeks, during which a standard diet composed of sugarcane, cactus (*Opuntia*) cladodes
35 and a commercial concentrate was provided *ad libitum*. Thereafter, the cows were randomly
36 assigned to the five dietary treatments in a replicated 5 × 5 Latin square design with 21-day
37 experimental periods (14 d for adaptation to diets and the last 7 d for sampling and data collection).
38 Diets consisted of different levels of GC for FFCG replacement (0; 25%; 50%; 75% and 100%)
39 based on DM. All diets contained similar proportions of cactus (*Opuntia stricta* [Haw]. Haw)
40 cladodes, sugarcane, and concentrate (32:34:34, on a DM basis). The FFCG was obtained by the
41 wet milling of corn, where the germ is separated by density, resulting in a high fat co-product with
42 high oxidative stability (Ingredion™). The chemical composition of forages and concentrates used
43 in the experimental diets is presented in the online Supplementary File (Table S1), while
44 proportions of ingredients and chemical composition of the diets are shown in Table S2. Diets
45 were formulated to meet energy and nutrient requirements of dairy cows producing 25 kg/d of fat-

46 corrected milk according to NRC (2001) and were fed *ad libitum* twice daily after morning and
47 afternoon milking as a total mixed ration (TMR). The amount of TMR provided to each cow was
48 adjusted daily to allow for 5 to 10 % of refusals.
49
50 *Sampling and data collection*
51 Individual feed intake was recorded daily by subtracting the amounts of feed offered from refusals.
52 From the 15th to the 21st day of each experimental period, samples of feed ingredients (cactus
53 cladodes, sugarcane and concentrate) and refusals were collected daily. Composite samples per
54 period (for feed ingredients) and per cow per period (for refusals) were formed and stored in plastic
55 bags at -20°C for subsequent chemical analysis.
56 To estimate the apparent digestibility of nutrients, fecal samples were collected directly from the
57 rectal ampoule of the animals, once a day, between the 16th and 20th days of each experimental
58 period, at 6:00, 8:00, 10:00, 12:00 and 14:00, respectively (Detmann *et al*., 2012). Then, the
59 samples were composed and homogenized by animal and period.
60 On the last day of each experimental period, four hours after morning feeding, spot urine samples
61 were collected from all cows during urination stimulated by vulvar massage. A 10 mL aliquot was
62 filtered through gauze, diluted in 40 mL of H_2SO_3 (0.036 N) and stored at -2°C for quantification
63 of allantoin, nitrogen, uric acid and creatinine concentrations.
64 The cows were milked twice a day (6:00 a.m. and 3:00 p.m.), and milk production was recorded
65 between the 15th and 21th days of each trial period. On the 18th and 19th day of each experimental
66 period, composite milk samples from morning and afternoon milking were collected in 50-mL
67 flasks containing Bronopol® as a preservative and analysed for protein, fat, lactose and total solids

content. Another 10 mL aliquot of milk was deproteinized with 5-mL of trichloroacetic acid (25%), filtered and stored at -20°C for allantoin analysis.

Analytical procedures

Samples of feed ingredients, refusals and feces collected throughout the study were thawed, pre-dried at 55ºC for 72h in a forced ventilation oven, ground in a knife mill (Model Thomas Wiley Co., Swedesboro, NJ) fitted with a 1-mm screen sieve, and analyzed for DM (method 934.01), organic matter (OM, method 930.05), ash (method 942.05), crude protein (CP, method 968.06) and ether extract (EE, method 920.39) according to AOAC (2005). Starch content of feed ingredients were determined according to AOAC method 996.11 (AOAC, 1995) with modifications reported by Walter *et al.* (2005). Neutral detergent fiber (NDF) was determined according to Mertens (2002) using a heat-stable alpha-amylase without sodium sulphite and corrected for residual ash. The NDF value was also corrected for non-protein nitrogenous compounds as described by Licitra *et al.* (1996).

The total fecal excretion was estimated using the indigestible neutral detergent fiber (iNDF) as an internal marker, and the feces, feed and orts iNDF content were obtained after 288 h of ruminal incubation time (Detmann *et al*., 2012).

Uric acid and creatinine analyzes were performed at the Clinical Pathology Laboratory of the Department of Veterinary Medicine at UFRPE, using commercial kits (LABTEST®), and the reading was performed on a semi-automatic biochemical analyzer (Labtest Diagnóstica, Lagoa Santa, Brazil). Urine allantoin analyzes were performed using the colorimetric method (Chen & Gomes, 1992). Urine nitrogen assessment was performed by the Kjeldahl distillation method according to INCT-CA method no. N-001/1 (Detmann *et al.,* 2012).

91 The concentrations of fat, protein, lactose and total solids in milk were analyzed by mid-infrared

92 spectrometry (Bentley Instruments, Bentley FTS, Chaska, MN, USA) according to the

93 International Dairy Federation protocols for whole milk samples (ISO 9622/ IDF 141, 2013).

94

95 *Calculations*

96 Non-fiber carbohydrates (NFC) were calculated according to Hall (2000). The diets' TDN content

97 and its conversion in lactation net energy (NEl) were estimated according to NRC (2001).

98 Daily total urinary volume was estimated through the relation of daily urinary excretion of

99 creatinine, using the observed values of creatinine concentration in urine as described by Valadares

100 *et al.* (1999). The daily urinary excretion of creatinine was based on 24.05 mg/kg of BW of

101 creatinine (Chizzotti *et al.,* 2008).

102 The microbial protein synthesis was estimated according Chen & Gomes (1992), considering

103 recovery of absorbed purines of 0.85 (Verbic *et al.,* 1990) and an endogenous contribution to the

104 excretion of purines as recommended by Gonzalez-Ronquillo *et al.* (2003).

105 The milk N was quantified using milk total protein (MTP/6.38), and the analyze allantoin in milk,

106 we used the colorimetric method as described by Chen & Gomes (1992). The nitrogen balance

107 was obtained by calculating the difference between total nitrogen intake and nitrogen excreted in

108 feces (N-feces), urine (N-urine) and milk (N-milk).

109 The milk yield corrected for energy (ECMY) was estimated as the equation ECMY=[(0.327 × kg

110 of milk) + (kg of fat × 12.95) + (kg of protein × 7.2)] (Tyrell & Reid, 1995).

111

112 *Test II*

113 *In vitro incubations*

114 The *in vivo* methane production was estimated from an *in vitro* assay based on a fully automated
115 gas production system and using kinetic parameters estimated by a mechanistic dynamics rumen
116 model developed by Ramin & Huhtananen (2012). The *in vitro* assay was performed at the
117 Swedish University of Agricultural Sciences, Umeå, Sweden.
118 Three 48 h incubations were performed. Before each incubation, samples of all experimental
119 ingredients (Table S1) were dried at 55°C and ground to 1 mm. The ingredients were corrected for
120 DM and then weighed in the proportions of the experimental diets inside glass bottles of serum
121 with a total substrate of 1006±14 mg.
122 The rumen fluid used in the incubations was obtained from two lactating Swedish Red cows fed a
123 diet composed of 60% roughage and 40% concentrate. The animal handling for this trial was
124 approved by the Swedish Ethics Committee on Experimental Animals (Dnr A 32-16). The rumen
125 fluid (average pH 6.3) was collected individually from each cow via cannula, filtered through
126 cheese cloth in two layers and placed in preheated thermos bottles previously treated with CO_2.
127 Similar proportions of the rumen fluid were added to a mineral buffer solution added to
128 PeptoneTM (pancreatic digested casein; Merck, Darmstadt, Germany) according to the
129 methodology described by Menke & Steingass (1988).
130 Serum bottles sealed with caps were placed in a water bath with gentle and constant agitation at
131 39°C and treated with CO_2 to ensure an anaerobic environment. Finally, 60 mL of buffered rumen
132 fluid solution was individually injected into the bottles and the gas channels were connected,
133 initiating incubation.
134 This process was repeated in the three incubations and all the diets tested finally had three
135 replicates (one in each incubation). Also, all incubations included blank bottles where there was
136 no addition of diet, only buffered rumen fluid for corrections of total gas and methane production.

137 The diets were randomly distributed in the bottles between the three incubations, avoiding
138 repetition of the diets in the gas reading channels. Thus, bottles and incubations were added to the
139 statistical model.
140

141 *Evaluation of pH and ruminal ammoniacal nitrogen*

142 The ruminal pH was measured at the end of the incubations (48h) as well as the collection of
143 ruminal fluid samples (0.6 mL) from the bottles. The rumen fluid samples were immediately stored
144 at -20 °C until the ammonia nitrogen (NH_3-N) analysis. The NH_3-N concentration was quantified
145 by the colorimetric method described by Chaney & Marbach (1962) using AutoAnalyzer 3 (SEAL
146 Analytical Ltd., Mequon, WI, USA).
147

148 *Evaluation of in vitro gas production and sampling*

149 The gas production system (Gas Production Recorder, GPR-2, Version 1.0 2015, Wageningen UR)
150 was set to take readings every 12 minutes and corrected for normal air pressure condition (101.3
151 kPa). The *in vitro* CH_4 measurement was performed according to Ramin & Huhtanen (2012), in
152 which gas samples were collected during the incubation period (0.2 mL) for each bottle at 2, 4, 8,
153 24, and 48h. The CH_4 concentration was determined with a gas chromatograph (Varian Star 3400
154 CX, Varian Analytical Instruments, Walnut Creek, CA, USA) equipped with a thermal
155 conductivity detector.
156

157 *Calculations and prediction of methane production in vivo*

158 The average gas production of blank bottles (correction bottles) in each incubation was deducted

159 from the gas production of the samples. The predicted in vivo methane production was calculated

160 as described by Ramin & Huhtanen (2012).

161 The predicted *in vivo* methane production can be expressed in g/kg DM from: CH_4 (g/kg DM) =

162 CH_4 (L of CH_4/kg of DM intake) × 1 (L) / 22.4 (L/mol) × 16.04 (g/mol), where 22.4 is the volume

163 of gas and 16.04 is the molar mass of CH_4.

164 The total daily methane production in grams was then estimated from the DM intake (data obtained

165 in Test I, total of 32 observations) × CH_4 production (data obtained in Test II, mean value of

166 methane production for each experimental diet; Table 4). Finally, the methane intensity (g of

167 CH_4/kg of ECMY) was calculated by the daily methane production (values obtained from the two

168 tests)/ ECMY (data obtained from Test II).

169

170 *Statistical analysis*

171 Data regarding trial I were analyzed using the PROC GLIMMIX of SAS (SAS, 2012) according

172 to 5 x 5 Latin square design balanced for carryover effects, using the following model:

173 $$Y_{ijkl} = \mu + T_i + Q_j + P_k + (A / Q)_{lj} + (T * Q)_{ij} + \varepsilon_{ijkl}$$

174 Where: Y_{ijkl} = dependent variable ijkl; μ = overall average; T_i = fixed treatment effect i; Q_j = fixed

175 square effect j; P_k = random period effect k; $(A / Q)_{lj}$ = random effect of animal l in the square j;

176 $T * Q_{ij}$, = effect of the interaction treatment i and square j; $\varepsilon_{ijk} \sim N(0,\sigma_2 e$ = random residual error.

177 The data regarding trial II were analyzed through the following model:

178 $$Y_{ijk} = \mu + T_i + I_j + G_k + \varepsilon_{ijk},$$

179 Where: Y_{ijk} = dependent variable ijk; μ = overall average; T_i = treatment i; I_j = incubation j; G_k =

180 bottle k; and $\varepsilon_{ijk} \sim N(0,\sigma 2e)$ random residual error.

181 Linear and quadratic effects of increasing dietary FFCG levels were tested by orthogonal
182 polynomial contrasts. We assumed significance effect when $\alpha \leq 0.05$.
183

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237

238 **Table S1**. Chemical composition (% of DM, unless otherwise stated) of feed ingredients used in

239 the experimental diets

Item	Sugarcane	Cactus cladodes	Soybean meal	FFCG[a]	Ground corn
Dry matter (g/kg as-fed)	275	126	897	949	902
Organic matter	969	880	880	980	984
Crude protein	21.5	49.8	480	100	90
Ether extract	5.26	14.6	32.6	490	32
Neutral detergent fibre[b]	450	257	133	247	135
Non-fiber carbohydrates[c]	491	555	296	130	727

240 [a] Full-fat corn germ

241 [b] NDF assayed with a heat-stable amylase and corrected for ash and nitrogenous compounds.

242 [c] Calculated as described by Hall (2000).

243 [d] Not detected.

244

245 **Table S2.** Proportion of ingredients and chemical composition (g/kg DM, unless otherwise

246 stated) of experimental diets

Item	Replacement levels of GC for FFCG (%)				
	0	25	50	75	100
Ingredients					
Sugarcane	320	320	320	320	320
Cactus cladodes	340	340	340	340	340
Soybean meal	147	148	149	150	151
Ground corn	160	120	80	40	-
Full-fat corn germ (FFCG)	-	40	80	120	160
Urea+Ammonium sulphate[a]	13	12	11	10	9
Premix[b]	15	15	15	15	15
Salt	5.0	5.0	5.0	5.0	5.0
Chemical composition					
Dry matter (g/kg, as-fed)	236	236	234	237	236
Organic matter	918	917	917	917	917
Crude protein	143	141	140	139	137
Ether extract	16.6	34.7	53.1	71.7	89.8
Neutral detergent fibre[c]	273	278	282	286	291
Starch[d]	193	165	136	108	80
Non-fibre carbohydrates[e]	442	421	400	379	358
Net energy, Mcal/kg MS[f]	1.65	1.63	1.72	1.75	1.73

247 [a] 9:1 ratio base on fresh matter.

248 [b] Commercial supplement containing the following minerals and vitamins (per kg): 205 g Ca, 60 g P, 15 mg Co, 700
249 mg Cu, 10 mg Cr, 700 mg Fe, 40 mg I, 1,600 mg Mn, 19 mg Se, 2,500 mg Zn, 600 mg F, 400,000 UI vitamin A,
250 2,400 UI vitamin E and 1,000 mg monensin.
251 [c] NDF assayed with a heat-stable amylase and corrected for ash and nitrogenous compounds.
252 [d] Values calculated using the starch content of individual feed ingredients and their proportions in the diets.
253 [e] Calculated as described by Hall (2000).
254 [f] Calculated according to NRC (2001).
255
256