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1 **Low degradable protein supply to increase nitrogen efficiency in lactating dairy**
2 **cows and reduce environmental impacts at barn level**

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14

15 **Short title: Nitrogen excretion and gas emissions in dairy cow**

16

17 **Abstract**

18 Generally, less than 30% of dairy cattle's nitrogen intake is retained in milk. Large
19 amounts of nitrogen are excreted in manure, especially in urine, with damaging
20 impacts on the environment. This study explores the effect of lowering dietary
21 degradable nitrogen supplies - while maintaining metabolisable protein - on dairy
22 cows' performance, nitrogen use efficiency and gas emissions (NH₃, N₂O, CH₄) at
23 barn level with tied animals. Two dietary N concentrations (CP: 12 %DM for LowN; 18
24 %DM for HighN) were offered to two groups of three lactating dairy cows in a split-
25 plot design over four periods of two weeks. Diets were formulated to provide similar

26 metabolisable protein supply, with degradable N either in deficit or in excess (PDIN of
27 84 and 114 g/kgDM for LowN and HighN respectively). Cows ingested 0.8 kg DM/d
28 less on the LowN diet, which was also 2.5% less digestible. Milk yield and
29 composition were not significantly affected. N exported in milk was 5% lower (LowN:
30 129 gN/d; HighN: 136 gN/d; $P < 0.001$) but milk protein yield was not significantly
31 affected (LowN: 801 g/d; HighN: 823 g/d; $P = 0.10$). Cows logically ingested less
32 nitrogen on the LowN diet (LowN: 415 gN/d; HighN: 626 gN/d; $P < 0.001$) resulting in
33 a higher N use efficiency (N milk / N intake; LowN: 0.31; HighN: 0.22; $P < 0.001$). N
34 excreted in urine was almost four times lower on the LowN diet (LowN: 65 gN/d;
35 HighN: 243 gN/d; $P < 0.001$) while urinary urea N concentration was 8-fold lower
36 (LowN: 4.6 g/L; HighN: 22.9 g/L; $P < 0.001$). Ammonia emission (expressed in g/h in
37 order to remove periods of the day with potential interferences with volatile molecules
38 from feed) was also lower on the LowN diet (LowN: 1.03 g/h/cow; HighN: 1.25
39 g/h/cow; $P < 0.05$). Greenhouse gas emissions (N_2O and CH_4) at barn level were not
40 significantly affected by the amount of dietary N. Offering low amounts of degradable
41 protein with suitable metabolisable protein amounts to cattle improved nitrogen use
42 efficiency and lowered ammonia emissions at barn level. This strategy would
43 however need to be validated for longer periods, other housing systems (free stall
44 barns) and at farm level including all stages of manure management.

45

46 **Keywords:** dairy cattle, nitrogen balance, urea, ammonia, greenhouse gas

47

48 **Implications**

49 In dairy systems, many farmers tend to secure the protein supply with “over
50 supplementation” to maximise milk production per cow, leading to higher feed costs

51 and N excretion. In a context of volatile input-output prices (Insee, 2015), together
52 with a societal demand to reduce environmental impacts, more efficient feeding
53 strategies are required. This study shows that better nitrogen use efficiency can be
54 achieved for dairy cattle fed with low amounts of degradable protein, leading to
55 reduced impacts on the environment, with a modest reduction in milk protein in the
56 short term.

57

58 **Introduction**

59 In Europe, animal production has been, and still is, encouraged to become more
60 productive (public policy measures such as tariffs and export subsidies) in order to
61 meet a growing consumer demand: e.g. the demand for meat and milk in 2050 is
62 projected to grow by 73% and 58% respectively compared with 2010 (FAO, 2011).

63 Intensive systems are often characterised by high inputs of nitrogen, which are
64 mostly excreted. This is not without consequence for global environmental and health
65 issues related to the emission of greenhouse gases (GHG) and other pollutants like
66 ammonia (NH₃; European Environment Agency, 2009, Gerber *et al.*, 2013).

67 Dairy cattle consume high amounts of soybean meal (Institut de l'élevage, 2011),
68 mainly imported from South-America and other parts of the world (Peyraud *et al.*,
69 2012), known to be associated with land use change and concomitant GHG
70 emissions (Gerber *et al.*, 2013, Pellerin *et al.*, 2013). Generally less than 30% of the
71 N intake is retained in the produced milk, and the remainder is typically excreted
72 roughly equally between faeces and urine (Castillo *et al.*, 2000, Calsamiglia *et al.*,
73 2010). In urine, nitrogen is principally found in the form of urea, which is rapidly
74 hydrolysed to ammonium (NH₄⁺) by urease enzymes present in faeces and soil. This
75 ammonium may then volatilise in the form of ammonia depending on temperature

76 and air velocity. Alternatively, it may be oxidised into nitrites and nitrates through a
77 two-step nitrification process, leading to emissions of intermediate compounds such
78 as nitric oxides (NO_x) and nitrous oxide (N₂O; Petersen *et al.*, 2013). Reducing
79 nitrogen excretion hence appears critical to reduce environmental impacts.

80 Lowering the amount of nitrogen consumed by the animals generally results in a
81 higher proportion of N exported to milk and decreased N excretion (Castillo *et al.*,
82 2000, Kebreab *et al.*, 2001). In the INRA feeding system, the metabolisable protein
83 (PDIE, protein digested in the small intestine when rumen-fermentable energy is
84 limiting; INRA, 2007) is used to calculate animal protein balance, assuming an
85 efficiency of 64% to transform PDIE into milk protein. Moreover, the requirements in
86 terms of degradable nitrogen for microbial protein in the rumen are considered to be
87 fulfilled when PDIN (protein digested in the small intestine when rumen-fermentable
88 nitrogen is limiting; INRA, 2007) equals or exceeds PDIE concentration in the diet.

89 Two strategies could then be used to reduce N excretion: 1/ improve protein use
90 efficiency by decreasing metabolisable protein supplies in relation to energy from the
91 diet (PDIE / UFL, milk feed unit, 1 UFL = 7.106MJ of net energy for lactation; INRA,
92 2007); 2/ favour urea reuse by a deficit in degradable protein in the diet ((PDIN –
93 PDIE) / UFL). A decline of PDIE intake was shown to reduce milk yield
94 (Cantalapiedra-Hijar *et al.*, 2014) and milk protein concentration (Monteils *et al.*,
95 2002) with a curvilinear response (Vérité and Delaby, 2000). On the other hand,
96 reducing PDIN available for microbes stimulates urea reuse for microbial synthesis
97 (Reynolds and Kristensen, 2008), resulting in lower N excreted in urine without
98 affecting protein synthesis and animal performance (Cutullic *et al.*, 2013), but with a
99 risk of decreasing microbial activity. Moreover, a lower amount of highly degradable
100 protein in the diet has been shown to modify the partition of N excreted between

101 urine and faeces, likely due to more efficiently captured N in the rumen (Kebreab *et*
102 *al.*, 2001).

103 The reduction of urinary N excretion should result in lower amounts of urea in
104 manure likely to volatilise in the form of ammonia (Dijkstra *et al.*, 2013). Moreover,
105 transformation from ammonium to nitrate via nitrification is a source of N₂O
106 (Chadwick *et al.* 2011); a variation of ammonium in manure could therefore influence,
107 even if marginally, N₂O emissions at the building level. Finally, methane (CH₄)
108 emissions from animal housing are mainly caused by enteric fermentation, sensitive
109 to rate of OM degradability, type of VFA produced and efficiency of microbial
110 synthesis (Monteny *et al.*, 2006). A reduction in N content of grass due to low N
111 fertilisation was also predicted to increase enteric CH₄ emissions, through
112 modification of rumen degradation characteristics and associated changes in the
113 carbohydrate composition and degradability of the feed (Bannink *et al.*, 2010). The
114 potential benefit from lowering dietary nitrogen supplies in terms of ammonia and
115 nitrous oxide emissions could therefore be counter-balanced by increased methane
116 emissions.

117 This study therefore explores the effect of lowering PDIN supplies from a theoretical
118 excess to a theoretical deficiency, while maintaining PDIE, on dairy cows' N use
119 efficiency and performance as well as gas emissions (NH₃, N₂O, CH₄) at barn level.
120 From current knowledge in the literature, we hypothesised that, due to a high
121 possibility of urea reuse for microbial synthesis in lactating dairy cows, a deficit in
122 PDIN supply in the diet will 1/ induce higher N use efficiency without detrimental
123 consequences on animal performance (dry matter intake and digestibility, milk
124 production and composition, etc.); 2/ decrease N excretion, especially in urine,
125 resulting in lower urinary urea content as well as lower ammonia and nitrous oxide

126 emissions from manure. With the will to avoid pollution swapping, methane emissions
127 were also monitored to ensure that the modulation of the diet composition was not
128 associated with higher methane production at barn level.

129

130 **Material and Methods**

131 The experiment was conducted at the INRA experimental farm of Méjusseume near
132 Rennes, France, from September to October 2008.

133

134 *Treatments, animals, experimental rooms and experimental design*

135 Treatments consisted of total mixed rations offering either low (LowN; CP: 12% DM,
136 84 g PDIN/kgDM) or high (HighN; CP: 18% DM, 114 g PDIN/kgDM) dietary
137 degradable nitrogen. The rations were composed of a 80:20 mixture of maize
138 silage:concentrate and were given *ad libitum* (constant access to the trough; refusal
139 maintained between 0.05 up to 0.10 of feed offered) and individually twice a day (\approx
140 8:00am and 6:00pm). Diets were formulated to provide similar PDIE concentrations
141 (96 and 99 g PDIE/kg DM for LowN and HighN respectively), but with degradable N
142 either in deficit or in excess (PDIN/PDIE of 0.87 and 1.15 for the LowN and HighN
143 respectively; INRA, 2007). Diet compositions are given in Table 1. Six Holstein dairy
144 cows in late lactation (221 ± 32 days in milk) were used for the experiment (Mean
145 Live Weight = 617 ± 13 kg). All were in their second or later lactation and were not
146 pregnant. Three of them were fitted with a rumen cannula; fistulated and non-
147 fistulated cows were kept apart throughout the experiment to account for potential
148 gas losses through the cannula. Each group was housed in a mechanically ventilated
149 and hermetic experimental room (temperature ≈ 18 °C) with negative air-pressure so
150 as to ensure unique and constant air extraction through the extraction duct. Air
151 renewal was achieved by the controlled air-conditioning system. The two rooms were
152 equipped with tied stalls and mattresses covered with sawdust (except during urine
153 and faeces collection), which were renewed daily. The cows had continuous access
154 to water and were milked on site twice a day.

155 The experiment followed a split-plot design with two groups of cows (fistulated and
156 non-fistulated cows), two treatments (LowN and HighN), and two experimental rooms
157 (A and B) during four periods of two weeks (Table 2). All the cows received the two
158 diets and were housed successively and conversely in the two experimental rooms to

159 account for possible biases due to ventilation systems. For each period of two weeks,
160 the first four days were used to accustom the cows to their diet; animal performances
161 (DM intake, milk yield and composition) were measured from days 5 to 14. From
162 days 8 to 11, urine and faeces were totally collected to estimate diet digestibility and
163 N excretion. Gaseous measurements were continuously taken from days 5 to 14 so
164 as to compare periods with and without manure collection. The cows were weighed
165 at the beginning and the end of the experiment.

166

167 *Measurements and analyses*

168 *Feed sampling and intake* Offered and refused feed was weighed precisely and
169 sampled every day to determine DM concentration (80°C, 48 h) in order to assess
170 individual cow DM intake. From days 8 to 11 of each period, samples of feed were
171 collected daily: concentrate was dried (80°C during 48 h), maize silage (offered and
172 refused) was freeze dried. Average samples of concentrate and maize silage per
173 period, and per cow for refusals, were then ground with a 3-blade knife mill through a
174 0.8 mm screen. The OM concentration was determined by ashing for 6 h at 500 °C.
175 Total N concentration was assessed by the Dumas method (Association Française
176 de Normalisation, 1997). Feed and refusals ADF, NDF, and ADL were analysed using
177 the method described by van-Soest *et al.* (1991), using a neutral detergent solution
178 containing α -amylase without sulphite, on a Fibersac analyzer (Ankom Technology,
179 Fairport, NY), and were corrected for ash concentration. Water intake was recorded
180 individually every day by monitoring mechanical water meters. Dietary PDIE and
181 PDIN concentrations were calculated from the diet ingredients chemical composition
182 (Table 1), and predicted rumen degradability of CP and intestinal digestibility of
183 rumen undegraded diet CP (INRA, 2007).

184 On day 10 of each period, rumen liquor was collected from fistulated cows at 8:00am
185 (just before the morning ration distribution) and again at 9:00am, 10:00am, 11:00am,
186 12:00pm, 2:00pm, 4:00pm and 6:00pm (just before evening ration distribution) to
187 assess pH kinetics. Samples were then frozen to determine N-NH₄⁺ concentrations
188 later (Berthelot colour reaction method).

189 *Milk yield and composition* Individual milk yield was monitored each day throughout
190 the experiment. Morning and evening milk samples were collected three times a
191 week (each Monday, Wednesday and Friday) to analyse for true protein and fat via
192 infrared analysis using a Milkoscan 605 (Foss Electric, Hillerød, Denmark). An
193 additional fresh milk sample was collected in the morning and evening of day 10 of
194 each period to assess individual milk total N (Kjeldahl method).

195 *Digestibility, faeces and urine* Digestibility ($[\text{intake} - \text{faecal output}] / \text{intake}$) was
196 estimated from days 8 to 11 for each period. Faeces were collected individually in the
197 gutter behind the animals, and were weighed daily. Animals were equipped with
198 harnesses to collect urine separately. Urine was acidified with 500 ml of H₂SO₄ 20%
199 to prevent ammonia volatilisation and weighed daily. Samples were collected every
200 day for each cow, and average samples were frozen every week in order to analyse
201 faecal DM, OM, and total-N (Dumas method; Association Française de
202 Normalisation, 1997). Urea-N was assessed in urine using colorimetric enzymatic
203 reaction with a multi-parameter analyser (KONE Instruments 200 Corporation,
204 Espoo, Finland).

205 *Uremia* In order to depict urea variations in relation to the diet, uremia was assessed
206 just before and 3 hours after the morning ration distribution. Even if urea
207 concentrations in blood and milk are correlated when measured at short time
208 intervals (Gustafsson and Palmquist, 1993), blood was preferred to milk as it was

209 easier to sample at both times. Blood samples were collected from the tail vein once
210 per period (day 10) at 8:00am and at 11:00am to assess plasmatic uremia (KONE
211 Instruments 200 Corporation, Espoo, Finland).

212 *Gas emissions measurements* Air samples were continuously collected in each
213 isolated room at air entrance and air extraction ducts so as to calculate a gradient.
214 Measurements of gas concentrations were performed on the last 10 days of each
215 period, therefore including periods with and without urine and faeces collection. An
216 infra-red photo acoustic analyser (INNOVA model 1312, Air Tech Instruments,
217 Ballerup, Denmark) was used coupled with a sampler-doser (INNOVA 1303). Air
218 samples were withdrawn from the experimental rooms into the analyser through 3
219 mm PTFE (Teflon®) sampling lines protected with dust filters, insulated and heated
220 to avoid water condensation within the sampling tubes. The analyser was fitted with
221 six filters, enabling the concentrations of six gases (NH₃, CO₂, CH₄, N₂O, SF₆ and
222 H₂O) to be sequentially measured. The analyser sampled air with a 2 min interval
223 between measurements, each location being analysed during 15 min, and a
224 computer was used to record concentrations. The instrument was span-calibrated
225 with known concentrations by the manufacturer before the experiment. The
226 instrument internally corrected for signal interferences from gases measured (optical
227 filters/detection limit: NH₃ 973/0.2 ppm; CO₂ 982/1.5 ppm; CH₄ 969/0.4 ppm; N₂O
228 985/0.03 ppm). However, for NH₃, non-compensated interferences were detected by
229 unexpected variations in gas concentration (two important peaks in NH₃
230 concentrations associated with feeding phases). As was recently highlighted by
231 Hassouna *et al.* (2013) under the same experimental conditions, these NH₃
232 oscillations were due to interferences with ethanol and acetic acid emitted by mixed
233 rations (maize silage in particular). We noticed that 6 h after the meal distribution the

234 quantity of feed remaining in the trough was close to the quantity of refusals (0.05-
235 0.10 of feed offered); from that time, interference was probably negligible. NH₃
236 emission measurements were therefore only considered between 12:00am and
237 7:00am and between 2:00pm and 5:00pm. The flow rate in each experimental room
238 was determined with the tracer ratio method using the constant dosing approach
239 (Baptista *et al.*, 1999). Sulphur hexafluoride (SF₆) was continuously injected through
240 PTFE sampling lines directly in the air entrance duct equipped with a mixing system
241 to homogenise air and SF₆. The amount of SF₆ released at each time step was
242 determined by the sampler-doser (INNOVA 1303). Ventilation rate (Q, in m³/h/cow)
243 was calculated as a function of time (t) from the rate of tracer release (ϕT) in m³/h
244 and the indoor tracer concentration (CT inside) in mg/m³ after correction for the
245 background concentration of the tracer (CT outside) (Demmers *et al.*, 2001):
246
247 Indoor (ambient) and outdoor (air entrance) temperature and moisture were
248 monitored using thermo-hygrometers while collecting air samples. Temperature,
249 moisture, flow rates and gas concentrations were expressed as mean values per h.
250 Gas emissions were calculated by multiplying the ventilation rate (m³/h/cow) by gas
251 concentrations (air extraction concentrations corrected by air entrance
252 concentrations, in mg/m³). Methane and nitrous oxide emissions were expressed as
253 cumulated gas emissions per cow per day; ammonia emissions per cow were
254 averaged at the hourly rate for the periods of the day when interferences were
255 expected to be negligible (i.e. between 12:00am and 7:00am and between 2:00pm
256 and 5:00pm).
257

258 *Statistical analyses*

259 For each period, only the last 10 days were considered for measurements, as the
260 first 4 days were used to adapt the animals to experimental conditions. For animal
261 performance and N balance variables, data was averaged per period for each
262 individual (N = 24) in order to overcome the temporal correlation between daily
263 measurements. Statistics were performed using the linear procedure of SAS 9.4
264 (PROC GLM; SAS Enterprise Guide v6.1 2013, SAS Institute Inc., Cary, NC, USA)
265 following the statistical model below:

$$266 Y_{ijklm} = \mu + Nconc_i + Room_j + Period_k + Group_l + Cow(Group)_{m(l)} + e_{ijklm},$$

267 where Y_{ijklm} is the studied variable; μ is the average; $Nconc_i$ is the diet degradable N
268 concentration (LowN or HighN, 1 df); $Room_j$ is the experimental room housing the
269 cows (A or B, 1 df); $Period_k$ is the period of two weeks (1 to 4, 3 df); $Group_l$ is the
270 group of cows (fistulated cows vs. normal cows, 1 df); $Cow(Group)_{m(l)}$ is the cow
271 nested in the group of cows (4 df); and e_{ijklm} is the error associated with each Y_{ijklm} . In
272 order to account for the split-plot design, the factor $Group_l$ was specifically tested
273 against the $Cow(Group)_{m(l)}$ error.

274 Hourly (NH_3) and daily gas emissions (N_2O , CH_4 , CH_4/kg DMI) were averaged per
275 experimental room and per period, identifying excreta (urine + faeces) collection and
276 no-collection periods (N = 16), and compared using the linear procedure of SAS 9.4
277 (PROC GLM; SAS Enterprise Guide v6.1 2013, SAS Institute Inc., Cary, NC, USA)
278 following the statistical model below:

$$279 Y_{ijklmn} = \mu + Nconc_i + Collection_j + Room_k + Period_l + Group_m + (Nconc \times Collection)_n +$$

280 e_{ijklmn}

281 where Y_{ijklmn} is the studied variable; μ is the average; $Nconc_i$ is the diet degradable N
282 concentration (LowN or HighN, 1 df); $Collection_j$ is the excreta collection status (yes

283 or no, 1 df); Room_k is the experimental room housing the cows (A or B, 1 df); Period_l
284 is the period of two weeks (1 to 4, 3 df); Group_m is the group of cows (fistulated or
285 not, 1 df); and e_{ijklmn} is the error associated with each Y_{ijklmn}.

286 Because of confounding effects, the interactions Nconc x Room and Nconc x Group
287 could not be tested in either model.

288

289 **Results and Discussion**

290 *Maintained animal performances and improved N use efficiency*

291 Compared with the HighN diet, animals that were fed low amounts of degradable N
292 ingested 0.8 kg DM/d less ($P < 0.001$) with a 2.5% reduction in DM digestibility ($P <$
293 0.001 ; Table 3); however, during the 8 weeks of experimentation, all the cows gained
294 weight ($+14 \pm 5$ kg). Nitrogen excreted in milk was 5% lower ($P < 0.001$), but milk
295 protein secretion was only 3% lower ($P = 0.10$; Table 3), the difference probably
296 being due to non-protein nitrogen content, higher in the HighN diet. Neither milk yield
297 ($P = 0.13$) nor milk composition ($P = 0.16$ and $P = 0.23$ for fat and protein
298 concentration respectively) were significantly affected by N supply; only milk fat yield
299 was reduced on the LowN diet (difference of 40 g/d, $P = 0.02$; Table 3). These results
300 corroborate those of Cutullic et al. (2013) where a reduction of degradable protein
301 supply in the diet (-10 g (PDIN-PDIE)/UFL; 12% CP) did not significantly affect milk
302 yield and protein milk yield for dairy cows compared with a balanced diet (PIDE =
303 PDIN). When both PDIN and PDIE supplies are reduced, the decrease in milk and
304 milk protein yields is generally more important, (e.g. -2 kg/d and -13 to 18% nitrogen
305 in milk; Monteils *et al.*, 2002, Cantalapiedra-Hijar *et al.*, 2014). In the present study,
306 PDIE concentrations of the diets were deliberately maintained equivalent between
307 treatments even if a small reduction in PDIE supply came from the decrease in DM

308 intake. A deficit of PDIE (under the threshold of 100 g PDIE/UFL) is known to rapidly
309 lead to important drops in performance (Vérité and Delaby, 2000). Moreover, in order
310 to obtain an optimal microbial protein synthesis and a satisfying diet digestibility,
311 microbes need a minimal and balanced amount of fermentable energy and
312 degradable protein in the rumen. Dairy cattle can tolerate a deficit of PDIN (down to
313 $(PDIN - PDIE)/UFL = -8$ g/unit UFL for low milk production; INRA, 2007) as microbes
314 in the rumen are able to use a certain amount of NH_4^+ -N recycled in the form of urea.
315 On the other hand, the excess of PDIN for the HighN diet ($(PDIN - PDIE)/UFL = 15$
316 g/unit UFL) resulted in higher amounts of nitrogen excreted compared with the LowN
317 diet, especially in urine ($P < 0.001$, Table 3). As cattle consumed much less nitrogen
318 in the low N diet (-33%) and exported quite similar quantities of nitrogen in milk (-
319 5%), they achieved considerably improved N use efficiency (from 22% up to 31%; P
320 < 0.001), which is consistent with the literature (Huhtanen and Hristov 2009).
321 Moreover, as both milk protein concentration and yield were not significantly affected
322 by the diet ($P > 0.05$, Table 3), it seemed that the animals were able to achieve a
323 better use of nutrients with a lower amount of feed ingested, as illustrated by the food
324 conversion ratio that tended to be higher for the LowN diet (1.24 vs. 1.22, $P = 0.05$,
325 Table 3). This could represent a certain economy for farmers and act as
326 compensation for the slight loss in milk production (Frank and Swensson, 2002). For
327 example, changing the diet from 16% to 14% CP by replacing soybean meal and
328 urea by grains was estimated to bring a benefit of 12 €/cow/year, provided balanced
329 supplies of PDIN and PDIE were maintained (taking into account milk yield and milk
330 protein concentration reductions in winter; Pellerin *et al.*, 2013). The recent inflation
331 of nitrogen-rich feed prices (Insee, 2015) should encourage farmers to promote low

332 degradable N diets, even more so if it results in improved protein self-sufficiency on
333 these farms (Pellerin *et al.*, 2013).

334

335 *Partitioning of nitrogen excretion and consequences for ammonia emissions*

336 As expected, nitrogen excreted in urine was substantially decreased with the LowN
337 diet ($P < 0.001$), representing as little as 30% of the total N excreted in manure (urine
338 + faeces) vs. 60% for the HighN diet. On the other hand, the amount of N excreted in
339 faeces was not significantly modified ($P = 0.36$, Table 3). This observation is
340 consistent with the literature for diets varying in CP concentration (Castillo *et al.*,
341 2000, Monteils *et al.*, 2002, Cantalapiedra-Hijar *et al.*, 2014). As already mentionned
342 by Kebreab *et al.* (2001), the proportion of N excreted in faeces in relation to N intake
343 increased (from 26% to 38%) when animals were fed diets with lower amounts of
344 highly degradable protein.

345 Ruminal kinetics showed a peak of NH_4^+ -N in rumen liquor after the morning ration
346 distribution. Ruminal NH_4^+ -N concentration was 4 times higher for the HighN diet at
347 09:00am (76 and 396 mg/L for the LowN and the HighN diets respectively, $P < 0.001$,
348 RMSE = 44; Figure 1) and stayed more elevated throughout the day ($P < 0.001$).

349 Despite this peak, ruminal pH was not significantly affected by treatment, either
350 before or after the meal ($P > 0.05$). Urinary and blood urea concentrations were
351 considerably lower for the LowN diet ($P < 0.001$, Table 3) leading to a large drop in
352 urinary urea excretion (47 g/d vs. 385 g/d for the LowN and HighN diets respectively,
353 $P < 0.001$). The clearance rate of urea (volume of blood cleared per unit of time) can
354 be calculated as the ratio between urinary urea excretion (g/d) and blood urea
355 concentration (g/L). In the present study, the clearance rate of urea was almost twice
356 as high for the HighN diet (36 L/h) as it was for the LowN diet (20 L/h). These values

357 are consistent with recent studies reporting that urea clearance was reduced from 41
358 to 27 L/h when the diet CP concentration of lactating cows decreased from 17% to
359 13% DM (Kristensen *et al.*, 2010) and from 35 to 25 L/h for decreasing urea ruminal
360 infusions (Rojen *et al.*, 2011). The large drop in urinary urea excretion for the LowN
361 diet was moreover accompanied by an important decrease in terms of the proportion
362 of urea N in urine (34% of total urinary N) compared to the HighN diet (76% of total
363 urinary N). Nitrogen deficiency in the LowN diet therefore led to nitrogen saving in the
364 kidney (Rojen *et al.*, 2011). As discussed by Kohn *et al.* (2005), keeping urea in the
365 blood may enable herbivores to recycle greater amounts of N in the gut compared
366 with omnivores, which explains how herbivores can survive on low amounts of low-
367 quality protein.

368 As expected, hourly NH₃ emissions were significantly decreased on the LowN diet
369 compared with the HighN diet (respectively 1.03 and 1.25 g NH₃/h/cow, $P = 0.01$,
370 Table 4). The 18% reduction does not seem very important given the large reduction
371 in urine volumes (1.5 times lower, $P < 0.001$) and urinary urea concentration (divided
372 by 5, $P < 0.001$) for the LowN diet vs. the HighN diet and when compared with the
373 literature. In controlled conditions, ammonia emission from manure (measured in flux
374 chambers) was for example 3 times lower when dietary CP decreased from 21% to
375 15% (Burgos *et al.*, 2010). At the dairy barn level, based on a large range of diets,
376 ammonia emissions were predicted to be reduced by 80% with a grass-based diet
377 with balanced amounts of rumen degradable protein (Monteny *et al.*, 2002).

378 However, as reported by Powell *et al.* (2008), ammonia emissions from tie-stall dairy
379 barns, where narrow gutter scrapers remove manure once or twice a day, are usually
380 lower than from free-stall barns where the emitting surface is much larger. In the
381 present study, the small reduction in ammonia emissions could therefore be viewed

382 as the result of the interaction between diet and a low emitting housing system. On
383 the other hand, the non-reduced ammonia emissions when excreta were collected
384 and acidified either as a single factor ($P = 0.44$, $N = 16$) or in interaction with the N
385 concentration of the diet ($P = 0.12$, $N = 16$) are surprising. However, the calculation of
386 mean hourly emissions excluding feeding periods - because of interferences - could
387 limit these interpretations.
388

390 Unaccounted N (not recovered either in manure or in milk) was 66 and 94 g N/d/cow
391 for the LowN and the HighN diets respectively, representing around 15% of N intake
392 for both treatments. These proportions are high but not unexpected when compared
393 with the literature. As pointed out by Cheng *et al.* (2011), the nitrogen balance
394 technique is difficult to implement and often leads to overestimations of N retention.
395 In a meta-analysis, Spanghero and Kowalski (1997) recorded an average N balance
396 of 39 g/d (ranging from -57 up to 205 g/d), representing 8% of N intake and 1/3 of N
397 contained in milk. More recently, Klevenhusen *et al.* (2010) reported N balances of up
398 to 26% of N intake (maize diet, 21% CP), and even if the average value reported in
399 the review of Spek *et al.* (2013) is only 2% of N intake (for EU trials), the high
400 standard deviation of 31 (N = 68) suggests high variations. In the present study,
401 considering that the cows were adult and non-pregnant, N retention could only be
402 linked to N accretion related to the energy balance of the animals. Calculations of the
403 animals' energy balance (based on UFL intake, mean body weight and fat corrected
404 milk yields as described in INRA, 2007) for each treatment, using the value of 6 g N
405 retained per UFL reported by Faverdin and Vérité (1998), make it possible to
406 estimate N retention in cows of 19 and 20 g N/d/cow respectively for the LowN and
407 the HighN diets. Nitrogen balances corrected for N retention were therefore 47 and
408 74 g N/d/cow, representing about 11% of N intake. Consequently, a significant part of
409 N ingested was probably lost during the recovery process or because of errors
410 associated with the measurement of N concentrations in the various samples.
411 Volatile N losses during urine collection should be prevented by the addition of a
412 strong acid (here H₂SO₄ 20%). In contrast, underestimation of faecal N by incomplete
413 collection of material or volatile losses of ammonia during collection and subsequent
414 drying of the samples could be important (e.g. -15% N in faecal samples after drying

415 compared to fresh faeces; Spanghero and Kowalski 1997). Echoing the words of
416 Reynolds and Kristensen (2008), mysteries of N balance still need to be solved
417 today.

418

419 *Consequences of N dietary manipulation for GHG emissions*

420 The dietary treatment did not affect N₂O ($P = 0.92$) and CH₄ emissions either
421 expressed in g/d or in g/kg DM intake (respectively $P = 0.65$ and $P = 0.41$; Table 4).

422 We could not identify any group effect (fistulated cows vs. normal cows) in measured
423 emissions of any of the gases concerned, suggesting that losses through the cannula
424 could not be detected, even for methane.

425 Urinary N is the principal source of N₂O emissions (Dijkstra *et al.*, 2013). In this
426 experiment, urine and faeces were deposited on small areas (gutters) and stayed for
427 short periods in the house before being scraped twice daily. Consequently, N₂O
428 emissions were very low (daily averages between 0.07 and 1.86 mg/m³) and
429 variations were difficult to detect for the photo acoustic IR gas analyser (detection
430 limit of 0.05 mg/m³).

431 Methane emissions at room level measured in this paper are rather high (> 550
432 gCH₄/d and > 26 gCH₄/kgDMI) when compared with the literature (19-22 gCH₄/kgDMI
433 for grass and maize silage diets varying in CP concentration measured in respiratory
434 chambers, Reynolds *et al.*, 2010b). In the present study, we cannot exclude that part,
435 even if marginal, of the methane at room level was produced by manure. Moreover,
436 gaseous emissions result from the product between gas concentrations and the
437 ventilation rate in the experimental room, both being associated with high
438 uncertainties (about 30% for the estimation of gas emissions from livestock;

439 Scholtens *et al.*, 2004). Biases being most likely the same for both treatments, they
440 should not affect the major conclusions of this study.

441 Bannink *et al.* (2010) reported that both observed and predicted enteric CH₄
442 emissions decreased with an increased dietary N:OM ratio, even if, as discussed by
443 the authors, variations in DMI may have affected these results. However, in the
444 literature, most papers were not able to link either the amount of total or digestible
445 protein consumed, nor N fertilisation levels to methane emissions (Reynolds *et al.*,
446 2010a, Podesta *et al.*, 2013), which would be consistent with our results that dietary
447 N modulation did not affect CH₄ emissions.

448 More generally, efforts to mitigate an emission can sometimes lead to higher
449 emissions of other pollutants, or of the same pollutant at a different stage; this is
450 referred to as pollution swapping (Monteny *et al.*, 2006). Reduced gas formation at
451 animal or manure level in the barn might be partially compensated by increased
452 gaseous emissions from manure in the following step of manure management
453 (Dijkstra *et al.*, 2011). In the future, more attention should be given to GHG and
454 ammonia mitigation options at farm level to account for effects on C and/or N flows
455 and associated gas emissions, considering all possible interactions at every stage of
456 the manure management continuum (Petersen *et al.*, 2013).

457

458 **Conclusion**

459 In the literature, most of the papers show that with less than 14% CP in the diet,
460 animal performance is severely reduced. This study demonstrates that dairy cattle
461 can tolerate, at least for periods of two weeks, important deficits of degradable
462 protein supply in maize based diets (down to 84 gPDIN/kgDM, 12% CP) without an
463 important drop in performances if metabolisable protein is maintained close to the

464 threshold of 100 gPDIE/UFL. The LowN diet might have favoured urea reuse for
465 microbial synthesis, compensating the deficit in degradable N in the rumen for milk
466 protein production. Excess intake of degradable nitrogen on the HighN diet (114 g
467 PDIN/kgDM, 18% CP) involved, on the contrary, higher uremia, resulting from greater
468 ruminal NH₃ production by microbes. This additional N was then lost in the form of
469 urea through urine, leading to increased NH₃ emissions. Offering low amounts of
470 degradable protein to cattle can therefore generate a win-win situation. On one hand,
471 low supplies of degradable N contributed to higher N use efficiency, at least on the
472 short term, with a limited reduction of performance that could be compensated by
473 lower amounts of feed ingested (potential economic gain). On the other hand,
474 impacts on the environment could be reduced via lower ammonia emissions
475 (acidification, eutrophication, human health, etc.). This strategy would however need
476 to be validated for longer periods of time, other housing systems (e.g. free stall
477 barns) and at farm level, including all stages of the manure management continuum.

478

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485

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- 631

632 **Table 1** Ingredients and nutrient composition of the two experimental diets varying in crude
 633 protein concentration fed to lactating dairy cows

Component, % DM unless noted	LowN ¹	HighN ¹
Ingredient		
Maize silage	79.7	79.5
Soybean meal mix ²	4.8	12.3
Soybean meal formaldehyde-treated	5.8	3.0
Mix concentrate ³	8.5	2.9
Urea	0.0	1.3
Minerals	1.0	1.0
Nutrients		
DM, % fresh matter	41.3	41.5
OM	94.6	93.5
Starch	30.5	27.9
CP	12.2	17.6
NDF	39.2	39.3
ADF	19.6	19.5
ADL	1.78	1.71
Fat	3.25	3.14
Ca	0.47	0.46
P	0.28	0.29
Feeding value		
UFL/kgDM ⁴	0.97	0.96
PDIE, g/kgDM ⁵	96.4	99.0
PDIN, g/kgDM ⁶	83.9	114.2
(PDIN – PDIE)/UFL	-12.8	15.8

634 ¹ LowN and HighN diets correspond to 12% and 18% of crude protein respectively

635 ² Soybean meal mix composed of 98% soybean meal and 2% of molasses

636 ³ Mix concentrate is composed of 25% wheat, 25% maize, 25% barley, 20% beet pulp, 3% molasses,
 637 1% vegetable oil, 1% NaCl

638 ⁴ UFL: Amount of net energy for milk production contained in 1 kg of a reference barley (87% of dry
 639 matter, 2,700 kcal of metabolisable energy), based on INRA feeding system (INRA, 2007)

640 ⁵ PDIE: True protein absorbable in the small intestine when rumen-fermentable energy is limiting in the
 641 rumen based on INRA feeding system (INRA, 2007)

642 ⁶ PDIN: True protein absorbable in the small intestine when rumen-fermentable nitrogen is limiting in
 643 the rumen based on INRA feeding system (INRA, 2007)

644

645 **Table 2** Description of the split-plot experimental design (LowN and HighN diets correspond to
 646 12% and 18% of crude protein respectively)

Room		Period			
		1	2	3	4
A	Cows Diet	Non-fistulated HighN	Non-fistulated LowN	Fistulated HighN	Fistulated LowN
B	Cows Diet	Fistulated LowN	Fistulated HighN	Non-fistulated LowN	Non-fistulated HighN

647

648 **Table 3** Influence of diet degradable N concentration on components of animal production,
 649 milk composition and nitrogen balance in late-lactating cows over the last 10 days of
 650 experimentation (least square mean, probability and root mean squared error of the
 651 model; N = 24)

	LowN ¹	HighN ¹	RMSE	P-value
DM intake kg/d	20.6	21.4	0.4	< 0.001
DM digestibility %	69.4	71.9	1.1	< 0.001
Milk yield kg/d	25.6	26.1	0.8	0.13
Milk fat g/kg	42.1	43.3	2.0	0.16
Milk fat yield g/d	1075	1115	38	0.02
Milk protein g/kg	31.4	31.7	0.6	0.25
Milk protein yield g/d	801	823	30	0.10
Water intake L/d	66.5	72.1	2.9	< 0.001
Urine L/d	10.3	16.8	1.6	< 0.001
Food conversion ratio (Milk yield / DM Intake)	1.24	1.22	0.03	0.05
N balance g/d	66	94	16	< 0.001
N intake g/d	415	626	10	< 0.001
N milk g/d	129	136	4	< 0.001
N faeces g/d	159	163	10	0.36
N urine g/d	65	243	19	< 0.001
N use efficiency %	31	22	1	< 0.001
Urinary urea g/L	4.6	22.9	1.1	< 0.001
Blood urea g/L ²	0.10	0.45	0.20	< 0.001

652 ¹ LowN and HighN diets correspond to 12% and 18% of crude protein respectively.

653 ² Mean of two samples (before and 3 h after the morning ration distribution)

654

655

656 **Table 4** Influence of diet degradable N concentration on ammonia and greenhouse gas
 657 emissions in late-lactating cows housed in tied stall barns (least square mean, probability
 658 and root mean squared error of the model; N = 16)

	LowN ¹	HighN ¹	RMSE	P-value
NH ₃ ² g/h/cow	1.03	1.25	0.15	0.01
N ₂ O g/d/cow	0.76	0.76	0.18	0.92
CH ₄ g/d/cow	586	567	74	0.65
CH ₄ g/kg DM intake	26.6	28.2	3.4	0.41

659 ¹ LowN and HighN diets correspond to 12% and 18% of crude protein respectively.

660 ² Ammonia emission measurements were only considered during periods of the day when
 661 interferences were expected to be negligible (i.e. between 12:00am and 7:00am and between 2:00pm
 662 and 5:00pm), and were averaged at an hourly scale.

663 **Figure captions**

664 **Figure 1** Ruminal liquor $\text{NH}_4^+\text{-N}$ concentration (mg/l) in late-lactating cows fed two
665 diets (open circles for the LowN diet at 12% CP; full squares for the HighN diet at
666 18% CP); statistical differences between diets are indicated: *** $P < 0.001$
667
668