Effect of carbohydrate source on microbial nitrogen recycling in growing rabbits

A. Belenguer[a,d,*], L. Abecia[a], A. Belanche[a], E. Milne[b], J. Balcells[a,c]

[a] Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Miguel Servet 177, 50013 Zaragoza, Spain

[b] Rowett Research Institute, Bucksburn, Greenburn Road, Aberdeen AB21 9SB, UK

[c] Departament de Producció Animal, Escola Tècnica Superior d'Enginyeria Agrària, Universitat Lleida, Alcalde Rovira Roure 199, 25198 LLeida, Spain

[d] Instituto de Ganadería de Montaña (CSIC – Universidad de León), Finca Marzanas s/n, 24346 Grulleros, León, Spain

* Corresponding author. Present address: Instituto de Ganadería de Montaña (CSIC – Universidad de León), Finca Marzanas s/n, 24346 Grulleros, León, Spain. Tel.: +34 987 317156; fax: +34 987 317161. E-mail address: a.belenguer@csic.es

Abstract

In rabbits, caecal fermentation relies to a large extent on the type of substrate available for bacteria. Therefore, in order to study the dietary effect of the source of carbohydrate on microbial nitrogen (N) absorption, thirty-two New Zealand growing male rabbits were randomly assigned to four diets formulated using two sources of structural carbohydrates (fibre), alfalfa hay (AH) and sugar beet pulp (SBP), combined with two sources of non-structural carbohydrates (starch), wheat or maize, at a constant fibre/grain sources ratio (0.80/0.20). Microbial N intake was estimated by preventing caecotrophy with a neck collar and, indirectly, by using urinary purine derivative (PD) excretion and microbial ^{15}N-lysine incorporation. No effect of diet on growth was detected (average growth rate 26.6±0.69 g/d), although dry matter (DM) intake was greater in animals fed diets with AH as main source of fibre than those receiving SBP (100.2 vs 90.1 g/d; $P<0.01$). Nonetheless, the latter diets were better digested and no significant differences were observed in digestible organic matter (OM) intake. Between sources of starch, digestibility of DM, OM and N was greater with wheat than maize ($P<0.05$). Microbial activity in the caecum was stimulated by SBP diets, as indicated by a greater volatile fatty acid concentration (89.6 vs 67.5 mmol/L; $P<0.01$) and a lower pH (5.7 vs 6.2; $P<0.001$). Significantly higher amino acid ^{15}N-enrichments in both caecotrophes and liver were observed with SBP diets and also in maize-fed rabbits when SBP was the main fibre source. However, microbial contribution to tissue amino acids (0.37±0.008) was not affected by the type of fibre.

Keywords: caecotrophy, caecum, fibre, lysine, starch, protein metabolism

1. Introduction

Monogastric herbivorous are hindgut fermenters, i.e. the fermentation compartment is located after the enzymatic digestion area and thus microbial biomass synthesised in the large intestine is mostly wasted with the faeces (Hörnicke, 1981). In order to recycle this nutritious material, lagomorphs, such as rabbits have developed a physiological mechanism, termed caecotrophy, involving ingestion of soft faeces (caecotrophes), originating from a selective retention process actively performed in the main fermentation compartment, the caecum, and proximal colon (Leng and Hornicke, 1976; Fioramonti and Ruckebusch, 1976; Hornicke, 1981). Caecal fermentation may be manipulated through the diet to improve the microbial contribution to rabbit protein nutrition. Potential nutritional factors include dietary components able to resist digestion in the small intestine and reach the caecum, such as structural carbohydrates (fibre) or resistant starch (Gidenne, 1997; Belenguer et al., 2002).

Quantification of the contribution of caecotrophy to the host amino acid (AA) metabolism has been limited by the accuracy of the methodology (Belenguer et al., 2008). Conventional procedure involves preventing caecotrophy using a neck collar to collect the soft faeces. Unfortunately, collar fitting in growing rabbits may alter dry matter intake (Carabaño et al., 2000a) and caecotrophes production (Fioramonti and Ruckebusch, 1976; Gidenne and Lebas, 1987; Gidenne and Lebas, 2006). Caecotrophy reached 26% of feed intake when estimated without a neck collar, but only 12 to 15% with the collar fitting procedure (Gidenne and Lebas, 2006). Another direct procedure, a serial slaughtering protocol (Gidenne, 1987), or indirect methodologies based on microbial markers utilization (internal - purine bases (PB), Balcells et al., 1998; or external - microbial ^{15}N-lysine, Belenguer et al., 2005) allow estimates of microbial-N

recycling *in vivo* without altering physiological behaviour, although the former technique produces only mean values and not individual estimations.

This study aimed to examine the effect of the dietary inclusion of two types of carbohydrates (starch or fibre), characterized by its high (wheat and sugar beet pulp (SBP)) or low (maize and alfalfa hay (AH)) digestive utilization, on caecal fermentation and microbial-N recycling, using either direct or indirect procedures.

2. Material and methods

Protocols and animal handling were approved by the "Comité Ético del Servicio de Biomedicina y Biomateriales" of the University of Zaragoza.

2.1. Animals and diets

Thirty-two New Zealand White newly weaned male rabbits (35-45 days), with a mean initial weight (W) of 0.91±0.017 kg were used. Animals were randomised among four experimental diets (8 animals per diet), housed individually in cages and submitted to a 12:12 h light:dark schedule, starting the light period at 8:00.

The four experimental diets were formulated to contain a similar energy (11.9-13.1 MJ digestible energy/kg dry matter, DM, estimated as described by Villamide et al., 2009), protein (170-185 g crude protein/kg DM), and lysine (10.2 mg/kg, estimated) content, using two sources of structural carbohydrates (fibre), alfalfa hay (AH) and sugar beet pulp (SBP). These fibrous ingredients were combined either with maize or wheat grain, as sources of non-structural carbohydrates (starch), at a constant fibre/grain sources ratio (0.80/0.20), i.e. containing about four times more fibre (SBP plus AH) than grain (wheat or maize). Diets were supplemented with ^{15}N-labelled NH_4Cl (0.7 %; $^{15}NH_4Cl$, 10^+ atom % ^{15}N ISOTEC, Inc USA) during the experimental period but not

during the adaptation period. Ingredients and composition of the four experimental diets are presented in Table 1. Diets were offered at 8:00 in the morning at a constant level (approximately 100 g/d; 90% *ad libitum* or 2.25 times maintenance requirements, defined as 410 kJ metabolizable energy/kg $W^{0.75}$/d; Xicatto and Trocino, 2010) for the whole experimental period and animals had free access to drinking water.

2.2. Experimental procedures

Each experimental period lasted for 35 days, 28 days for dietary adaptation and seven days for experimental measurements. Rabbits were penned individually during the adaptation period and in metabolism cages for the experimental trial. The following schedule was adopted on each experimental design: adaptation to the metabolic cage (days 1-2), digestibility measurements and urine collection (days 3-6), and collar fitting to prevent caecotrophy and allow collection of the soft faeces for the last 24 h (day 7), according to the method of Carabaño et al. (2010). A wooden round neck collar (50 mm i.d. and 270 mm e.d., weighing approximately 67 g) was fitted at 8:00 and maintained for 24 h. Animals were weighed at the beginning of the experiment and then once a week until the end of the trial when they were slaughtered between 9:00 and 12:00.

2.3. Sample collection

During the period of experimental measurements daily urine was collected individually under 1 M H_2SO_4 (50 ml/L, final pH<3), weighed, diluted (to 1 L), sampled (100 mL) and stored at –20ºC. Faeces were also collected daily, together with caecotrophes on day 7, then weighed and immediately frozen at –20ºC.

Animals were slaughtered by cervical dislocation, dissected and the caecum excised and weighed. Caecal content pH was measured with a glass electrode pH-meter.

Immediately afterwards, two samples of caecal contents were taken (1 g each), acidified with either 0.2 M HCl or 0.5 M H_3PO_4, and both stored at –20ºC for ammonia and volatile fatty acid (VFA) determination, respectively. The remaining caecal contents were weighed (20 to 50 g), diluted in a methylcellulose solution (9 g NaCl/L, 1 g methylcellulose/L) and chilled at 4ºC for 24 h to dislodge and isolate adherent bacteria as previously described (Belenguer et al., 2005). The resultant microbial pellet was frozen at -20ºC and then freeze-dried for subsequent analysis. Samples of liver were also taken and stored at -20ºC for ^{15}N-AA enrichment determination.

2.4. Chemical analyses

In feeds and faeces, either hard or soft, DM was determined by drying at 60ºC to constant weight. Organic matter (OM) was estimated by ashing samples at 550 ºC for 8 h. Nitrogen (N) was measured by the Kjeldhal method. Neutral and acid detergent fibre (NDF and ADF) and lignin were determined according to Van Soest et al. (1991) after an amylase pre-treatment. Caecal volatile fatty acids (VFA) concentration was analysed by gas chromatography, following the procedure described by Jouany (1982) and ammonia concentration by the method proposed by Chaney and Marbach (1962). Urinary purine derivatives (PD: allantoin, uric acid, hypoxanthine and xanthine) were analysed by reverse-phase HPLC (Balcells et al., 1992). Purine bases (PB: adenine and guanine) in feeds, bacterial extracts and soft faeces were determined by the same HPLC technique, with the modifications proposed by Martín-Orúe et al. (1995).

Amino acid ^{15}N-enrichments in caecotrophes and liver were measured by GC-C-IRMS as described previously (Belenguer et al., 2005).

2.5. Calculation and statistical analyses

Duodenal flow of PB was estimated from the urinary excretion of PD following the predictive model proposed by Balcells et al. (1998) as modified by Belenguer et al. (2008). The microbial lysine intake and the contribution of microbes to tissue lysine were estimated as described previously (Belenguer et al., 2005, 2008). Briefly, the microbial lysine intake was estimated assuming a true ileal digestibility of 0.82 (Carabaño et al., 2000b) and 0.88 (Storm et al., 1983) for dietary and microbial lysine, respectively. The microbial contribution to tissue lysine was calculated as the ratio of ^{15}N-enrichments in liver and microbial lysine. Data were analysed by ANOVA as a complete randomised design, following a 2 x 2 factorial structure, with the source of fibre (F; AH *vs* SBP), the source of starch (St; wheat *vs* maize) and their interaction as main effects. Analyses of ^{15}N-AA enrichment were done as a split-plot design, comparing main plot factors (fibre, F and starch, St) against the animal error term (Є1), whereas ^{15}N enrichment between substrates (Sub; liver and caecotrophes) nested within animals (A) and their interactions were compared against the residual error term (Є2). The model was as follows:

$$Y_{ijkl} = \mu + F_i + St_j + F\times St_{ij} + Є1_{ijk}\ [A(F\times St)] + Sub_l + F\times Sub_{il} + St\times Sub_{jl} + Є2_{ijkl}$$

When methodologies to estimate microbial N recycling were compared, data were analyzed using a similar split-plot design with method (M) used instead of substrate.

Data were analyzed using the using the MIXED procedure of the statistical software SAS (2004). The level of statistical significance was set at $P<0.05$.

3. Results

No significant effect of the interaction between fibre and starch (F x St) was observed in most studied parameters, and therefore only the effects of the main factors (fibre and starch) are presented in the tables unless otherwise stated.

165

166 *3.1. Digestive parameters*

167 Average growth rate was 26.5±0.69 g/d and final live weight was 1.90±0.024 kg, and
168 no differences among experimental groups were recorded (Table 2). The lower dry
169 matter intake in animals fed SBP diets (-10%) than in those receiving AH diets (P<0.01)
170 was counterbalanced by a higher DM and OM total tract apparent digestibility
171 (P<0.001). In consequence, digestible organic matter intakes were similar, although
172 NDF and N were also better digested in SBP (0.53 and 0.78, respectively) than in AH
173 diets (0.43 and 0.74; P<0.001).

174 Digestibility of DM, OM and N were higher for wheat than maize diets (0.70, 0.71
175 and 0.77 *vs* 0.68, 0.68 and 0.75, respectively; P<0.05 for DM and OM, and P<0.01 for
176 N), while there were no significant differences between starch sources for digestible
177 OM and N intake and NDF digestibility.

178

179 *3.2. Characterization of caecal fermentation*

180 Animals fed SBP had more fresh caecal contents (99.1 *vs* 80.6 g/d; P<0.05) and
181 higher empty caecum weight than those receiving AH diets (37.1 *vs* 32.9; P<0.01), but
182 there were no differences between starch sources (Table 2).

183 Rabbits with a larger caecum also showed higher total VFA concentrations (89.6 *vs*
184 67.5 mmol/L in rabbits fed SBP and AH diets, respectively; P<0.01) and a lower pH
185 (5.69 *vs* 6.18; P<0.01). Molar proportions of the main VFA did not differ between diets
186 (acetic, 0.79, butyric, 0.14, and propionic 0.06). Ammonia concentration averaged
187 3.90±0.431 mg/dL, and was independent of dietary treatment.

188 Caecotrophes showed a two-fold higher protein concentration (335 *vs* 144 g/kg DM)
189 but only half the NDF concentration (297 *vs* 567 g/kg DM) of hard faeces.

190

191 *3.3. Urinary excretion and duodenal flow of purine compounds*

192 Urinary PD was composed almost entirely of allantoin and uric acid (92 and 8%)
193 with trace amounts of xanthine and hypoxanthine. Allantoin and uric acid excretions
194 averaged 839.5±40.31 and 68.6±4.22 µmol/kg $W^{0.75}$, respectively, with both affected by
195 diet. Total PD excretion was greater in rabbits fed AH than in those receiving SBP as
196 the main source of fibre (1035.4 *vs* 780.7 µmol/kg $W^{0.75}$, respectively; $P<0.01$), but no
197 differences were detected between starch sources (Table 3). In addition, rabbits fed AH
198 diets showed a greater duodenal flow of PB than those fed SBP diets (1.55 *vs* 1.17
199 mmol/kg $W^{0.75}$; $P<0.01$) due to both the greater ingestion of dietary PB (0.83 *vs* 0.62
200 mmol PB/d for AH and SBP-fed rabbits, respectively) and a higher estimated duodenal
201 flow of microbial PB (1.07 *vs* 0.81 mmol/kg $W^{0.75}$ for animals fed AH and SBP diets,
202 respectively; $P<0.05$).

203

204 *3.4. Microbial ^{15}N-lysine incorporation*

205 Ingestion of the isotope (0.7 g/d $^{15}NH_4Cl$) resulted in enriched AA in caecotrophes
206 and liver (Table 4). For essential AA the isotope enrichments were always greater in
207 caecotrophes than in liver. With non-essential AA, however, there was no clear pattern,
208 with either lower (tyrosine, proline, aspartate, glutamate), similar (glycine), or higher
209 (alanine, serine) enrichments in liver.

210 The effect on ^{15}N-AA enrichment was always higher ($P<0.05$) for both caecotrophes
211 and liver in animals fed SBP compared to those receiving AH as the main source of
212 fibre. Effects of starch type were less pronounced, but for most amino acids, including
213 lysine, enrichments (atom % excess) were greater in maize-fed rabbits when SBP was
214 the main fibre source, whereas no significant differences were observed with AH, as

indicated by the significant fibre x starch interaction. For instance, this occurred for lysine either in caecotrophes (0.56 *vs* 0.50) or liver (0.22 *vs* 0.18; Table 4).

The contribution of microbial AA to tissues was estimated using lysine and threonine, AA that do not undergo amination or transamination, as described by Belenguer et al. (2005), although in the present study enrichments were measured in caecotrophes rather than caecal bacteria. Microbial contributions to liver lysine and threonine both averaged 0.37 (0.37±0.008 and 0.37±0.014, for lysine and threonine, respectively). Microbial lysine contribution to liver was not affected by the experimental treatment (Figure 1).

3.5. Microbial N recycling

Dry matter and N excretion through the caecotrophy process and microbial N recycling, estimated by the three approaches, are presented in Table 5. Average soft faeces excretion, determined by caecotrophe collection, was 12.7±0.71 g DM/d, with a high interindividual variation (from 5.8 to 22.7 g/d), and was unaltered by the experimental treatment.

Microbial N recycling values differed among estimation protocols, with the lower values obtained by direct collection (collar method; 0.50±0.032 g/d) compared to those derived from PD excretion (1.22±0.080 g/d) or ^{15}N-lysine incorporation (1.39±0.064 g/d; $P<0.001$). The effect of either the source of fibre or starch was not significant for any of the estimation methods. Residual variations (coefficient of variation, CV) were 37, 35 and 18%, for PD excretion, direct collection and microbial ^{15}N-lysine incorporation, respectively.

4. Discussion

4.1. Digestive and fermentation parameters

Animals adapted well to the experimental treatments, although growth rate was lower than in similar trials (26.6±0.69 *vs* 30-38 g/d; de Blas and Villamide, 1990; Gidenne and Bellier, 2000) where rabbits were fed *ad libitum*, while in our case feed supply was restricted (90% of *ad libitum* intake), in order to minimize individual feed intake variability.

SBP contains a greater proportion of hemicellulose plus water insoluble pectins (Gidenne, 2003) than AH. The AH has a greater degree of lignification, which may stimulate the transit of digesta through the digestive tract (Gidenne et al., 2001). These differences may explain variations in intake and digestibility and the high fermentation rates observed in SBP-fed rabbits (Gidenne et al., 2010), evidenced by a lower pH (-8%) and a higher VFA concentration (+33%; Fraga et al., 1991; Gidenne, 1997).

Likewise, maize and wheat grains differ in the physical structure of the starch granule. In maize the protein matrix that surrounds starch is thicker, which restricts the enzyme accessibility and reduces digestibility compared to wheat (Rooney and Pflugfelder, 1986; Blas and Gidenne, 2010).

4.2. Indirect approaches to estimate microbial contribution

PD excretion values were within the range described previously with similar animals and diets (Belenguer et al., 2008), although lower than values derived from older and heavier animals (Abecia et al., 2005). As previously observed (Belenguer et al., 2002), urinary PD was greater with AH than in SBP-fed animals, due to a higher intake of dietary PB. A similar effect was observed in total and microbial duodenal PB flow.

263 Ingestion of a ^{15}N-labelled NH_4Cl results in production of labelled microbial AA-N
264 (Belenguer et al., 2005) with a differential ^{15}N enrichment depending on metabolic
265 inflows from labelled or unlabelled sources (Atasoglu et al., 2004). In this regard, amino
266 acid enrichment in caecotrophes was greater in SBP-fed animals than in those animals
267 receiving AH diets. We might speculate that availability of dietary [non-labelled] N for
268 caecal microbes would be higher in AH-fed animals due to either more dietary resistant
269 protein (Gidenne and Ruckebusch, 1989) and/or greater protein ingestion. Alternatively,
270 SBP diets could have provided more fermentable fibre (such as hemicellulose) reaching
271 the caecum, which would elicit an increased level of microbial biosynthesis and
272 consequently recycling of endogenous labelled N sources.
273 Although starch is digested mainly in the small intestine (Merino and Carabaño,
274 1992; Carabaño et al., 1997), the resistant starch structure in maize granule (Blas and
275 Gidenne, 2010) may enhance the starch flow at the ileum (Gidenne et al., 2005) and the
276 microbial activity within the caecum. Thus caecotrophes seemed to show a higher level
277 of ^{15}N incorporation in maize than in wheat-fed rabbits, although this effect was
278 significant only with SBP as the main source of fibre (+12%), suggesting that the starch
279 effect could be enhanced by presence of fermentable fibre.
280 In tissues, the AA ^{15}N-enrichments rely on the contribution of microbial AA, derived
281 mostly from caecotrophy (Belenguer et al., 2005), plus amination/transamination
282 processes. Average tissue enrichments were lower in the essential AA, such as lysine,
283 that do not undergo transamination within tissues (Bender, 1985) and was then used to
284 estimate microbial contribution (mostly through caecotrophy) to AA absorption.
285 Differences in absolute AA enrichments in caecotrophes and tissues between fibre
286 sources (SBP *vs* AH, Table 4) were not reflected in the microbial contribution to liver
287 lysine, which seems to suggest that the ingested amount of caecotrophes relative to total

intake might be similar among treatments. If this is the case, variations between fibre sources in ^{15}N-AA enrichments in caecotrophes may be due to differential ^{15}N/^{14}N availability from either labelled (ammonia, body proteins) or unlabelled (dietary) sources in the caecum.

Regarding the source of starch, no significant effect was observed on microbial AA contribution to tissue lysine (Figure 1), despite the greater microbial ^{15}N incorporation in caecotrophes (and also in liver) in maize than in wheat-fed rabbits when SBP was the main source of dietary fibre.

The use of ^{15}N-lysine enrichments in caecotrophes instead of caecal bacteria may overestimate microbial contribution, since, as mentioned, ^{15}N-lysine enrichment in caecotrophes are lower (-18%) than in caecal bacteria. Even applying such a correction, the revised microbial contribution to tissue lysine (0.30) is still greater than values previously reported either with the same method in growing rabbits (0.22; Belenguer et al., 2005) and lactating does (0.23; Abecia et al., 2008), or with the caecotrophes collection technique in lactating does (0.18; Nicodemus et al., 1999). Differences among trials could be explained by different experimental conditions and in the present experiment the limited feed (and N) supply might have also enhanced the ability of the animals to recycle microbial protein.

4.3. Microbial N recycling

The average contribution of microbes to total protein intake differed among methodologies (29.3±1.47, 33.3±0.94 and 15.3±0.84% for PD, ^{15}N-lysine and caecotrophe collection procedures, respectively), confirming previous results from our group (Belenguer et al. 2005; Belenguer et al 2008). Similarly, the lowest values of microbial OM recycling (20.1±1.27, 22.7±0.90 and 11.3±0.63 g/d for PD, ^{15}N-lysine and caecotrophes collection procedures, respectively) or its contribution to total OM

intake (18.7±1.05, 21.2±0.54 and 11.6±0.62%, respectively) were observed with the conventional technique. Collar fitting may distress rabbits and reduce feed intake (Belenguer et al., 2002) and caecotrophes excretion (Gidenne and Lebas 1987; Belenguer et al., 2008), as a consequence of the altered nutritional behaviour. The DM intake was more variable and decreased with the collar (-21%), suggesting a lack of adaptation of the animals, which might explain the lower amount of caecotrophes collected (12.7 g DM/d) and the smaller microbial contribution to total N intake (15.3%) than the range reported by Villamide et al. (2010; 15-30 g DM/d or 17-29%).

The present study aimed, however, to investigate the effect of diet on microbial N recycling. In this regard, a previous study from our group (Belenguer et al. 2002), using similar diets (fibre/grain sources ratio, 0.80:0.20), showed an improvement of microbial N recycling (g/d) with AH diets in relation to SBP, using the conventional and the PD techniques, in rabbits fed *ad libitum*. Conversely, in the current study no significant differences were detected in microbial N recycling between treatments and thus the contribution of soft faeces to total DM (or N) was similar in all diets. Some aspects of the PB metabolism are still unclear in rabbits (Belenguer et al., 2008), and as far as the authors are aware, this is the first study of the effect of different sources of fibre on microbial N recycling using the ^{15}N-lysine incorporation method. With the soft faeces collection, different results on the effect of the type of fibre on microbial N excretion have been reported, with no variations between alfalfa and soya been hulls (Nicodemus et al. 2007) or differences (from 0.34 to 0.83 g microbial N/day) using a range of different fibre sources (paprika meal, olive leaves, alfalfa hay, NaOH-treated straw and hull from soybean and sunflower) at different inclusion levels (NDF varied from 33.1 to 78.7 % of DM; García et al., 2000). In the last case, however, differences were mostly explained by the low values obtained with straw and sunflower (0.34 g DM/d).

5. Implications: The utilization of two different sources of structural carbohydrates, AH and SBP, or two types of starch, wheat and maize, did not result in significant variations in microbial protein recycling. Nevertheless, our results suggest that the utilization of SBP, especially in combination with maize, as a dietary source that may provide a greater amount of carbohydrates to the hindgut, might increase microbial biosynthesis in the caecum, indicating a potential to improve microbial protein contribution. Further research would be necessary to elucidate this effect and make dietary recommendations for these ingredients for growing rabbits.

Acknowledgments

The authors gratefully acknowledge Dr Gerald Lobley from the Rowett Institute of Nutrition and Health (Aberdeen, UK) for his helpful assistance and suggestions in the preparation of the manuscript. Thanks are given to Animal Nutrition Department (Estación experimental del Zaidín, CSIC, Granada) for AA analysis and to the Servicio de Apoyo a la Investigación (Universidad de Zaragoza) for care of animals.

References

Abecia, L., Balcells, J., Fondevila, M., Belenguer, A., Calleja, L., 2005. Effect of therapeutic doses of antibiotics in the diet on the digestibility and caecal fermentation in growing rabbits. Anim. Res. 54, 307-314.

Abecia, L., Balcells, J., Fondevila, M., Belenguer, A., Holtrop, G., Lobley, G.E., 2008. Contribution of gut microbial lysine to liver and milk amino acids in lactating does. Br. J. Nutr. 100, 977-983.

363 Atasoglu, C., Guliye, A.Y., Wallace, R.J., 2004. Use of stable isotopes to measure de
364 novo synthesis and turnover of amino acid-C and -N in mixed micro-organisms from
365 the sheep rumen in vitro. Br. J. Nutr. 91, 253-262.
366 Balcells, J., Ganuza, J.M., Perez, J.F., Martín-Orúe, S.M., Gonzalez Ronquillo, M.,
367 1998. Urinary excretion of purine derivatives as an index of microbial-nitrogen
368 intake in growing rabbits. Br. J. Nutr. 79, 373-380.
369 Balcells, J., Guada, J.A., Peiro, J.M., Parker, D.S., 1992. Simultaneous determination of
370 allantoin and oxypurines in biological fluids by high-performance liquid
371 chromatography. J. Chrom. 575, 153-157.
372 Belenguer, A., Balcells, J., Fondevila, M., Torre, C., 2002. Caecotrophes intake in
373 growing rabbits estimated either from urinary excretion of purine derivatives or from
374 direct measurement using animals provided with a neck collar: effect of type and
375 level of dietary carbohydrate. Anim. Sci. 74, 135-144.
376 Belenguer, A., Balcells, J., Fondevila, A., Abecia, L., Solanas, E., 2008. Alternative
377 methodologies to estimate ingestion of caecotrophes in growing rabbits. Livest. Sci.
378 115, 13-19.
379 Belenguer, A., Balcells, J., Guada, J.A., Decoux, M., Milne, E., 2005. Protein recycling
380 in growing rabbits: contribution of microbial lysine to amino acid metabolism. Br. J.
381 Nutr. 94, 763-770.
382 Bender, D.A., 1985. Amino Acid Metabolism. Chichester: John Wiley & Sons.
383 Blas, E., Gidenne, T., 2010. Digestion of sugars and starch, in: de Blas, C., Wiseman, J.
384 (Eds.), The Nutrition of the Rabbit, 2nd ed., CAB International, Wallingford, UK, pp.
385 19-38.

386 Carabaño, R., García, A.I., Blas, E., Falcao, L., Gidenne, T., Pinheiro, V., 2000a.
387 Collaborative studies on caecotrophy in adult rabbits: effect of feed intake and
388 methodology. World Rabbit Sci. 8 Supl. 1, 153-160.
389 Carabaño, R., de Blas, J.C. , García, A.I., 2000b. Recent advances in nitrogen nutrition
390 in rabbits. World Rabbit Sci 8, Suppl. 1, 15–28.
391 Carabaño, R., Motta-Ferreira, W., de Blas, J.C., Fraga, M.J., 1997. Substitution of
392 sugarbeet pulp for alfalfa hay in diets for growing rabbits. Anim. Feed Sci. Technol.
393 65, 249-256.
394 Carabaño, R., Piquer, J., Menoyo, D., Badiola, I., 2010. The Digestive System of the
395 Rabbit, in: de Blas, C., Wiseman, J. (Eds.), The Nutrition of the Rabbit, 2nd ed., CAB
396 International, Wallingford, UK, pp. 1-18.
397 Chaney, A.L., Marbach, E.P., 1962. Modified reagents for determination of urea and
398 ammonia. Clin. Chem. 8, 131-143.
399 Chen, X.B., Hovell, F.D., Orskov, E.R., Brown, D.S., 1990. Excretion of purine
400 derivatives by ruminants: effect of exogenous nucleic acid supply on purine
401 derivative excretion by sheep. Br. J. Nutr. 63, 131-142.
402 de Blas, J.C., Villamide, M.J., 1990. Nutritive value of beet and citrus pulps for rabbits.
403 Anim. Feed Sci. Technol. 31, 239-246.
404 Fioramonti, J., Ruckebusch, Y., 1976. La motricité caecale chez le lapin. III. Dualité de
405 l'excretion fécale. Ann. Rech. Vet. 7, 281-295.
406 Fraga, M.J., Perez de Ayala, P., Carabaño, R., de Blas, J.C., 1991. Effect of type of fiber
407 on the rate of passage and on the contribution of soft feces to nutrient intake of
408 finishing rabbits. J. Anim. Sci. 69, 1566-1574.

409 Garcia, J., Carabaño, R., Pérez-Alba, L., de Blas, J.C., 2000. Effect of fiber source on
410 cecal fermentation and nitrogen recycled through cecotrophy in rabbits. J. Anim. Sci,
411 78, 638-646.
412 Gidenne, T., 1987. Influence de la teneur en lignines des aliments sur la composition
413 desdigesta et la production de caecotrophes chez le lapereau. Ann.Zootech, 36, 85-
414 90.
415 Gidenne, T., 1997. Caeco-colic digestion in the growing rabbit: impact of nutritional
416 factors and related disturbances. Livest. Prod. Sci. 51, 73-88.
417 Gidenne, T., 2003. Fibres in rabbit feeding for digestive troubles prevention: respective
418 role of low-digested and digestible fibre. Livest. Prod. Sci. 81, 105-117.
419 Gidenne, T., Arveux, P., Madec, O., 2001. The effect of the quality of dietary
420 lignocellulose on digestion, zootechnical performance and health of the growing
421 rabbit. Anim. Sci. 73, 97–104.
422 Gidenne, T., Bellier, R., 2000. Use of digestible fibre in replacement to available
423 carbohydrates: Effect on digestion, rate of passage and caecal fermentation pattern
424 during the growth of the rabbit. Livest. Prod. Sci. 63, 141-152.
425 Gidenne, T., García, J., Lebas, F., Licois, D., 2010. Nutrition and feeding strategy:
426 Interactions with pathology, in: de Blas, C., Wiseman, J. (Eds.), The Nutrition of the
427 Rabbit, CAB International, Wallingford, UK, pp. 179-199.
428 Gidenne, T., Lebas, F., 1987. Estimation quantitative de la caecotrophie chez le lapin en
429 croissance: variations en fonction de l'âge. Ann. Zootech. 36, 225-236.
430 Gidenne, T., Lebas, F., 2006. Feeding behaviour in rabbits. In: Feeding in domestic
431 vertebrates. From structure to behaviour. V. Bels Eds. CABI publishing,
432 Wallingford, UK, chapter 11 pp. 179-209.

433 Gidenne, T., Ruckebusch, Y., 1989. Flow and passage rate studies at the ileal level in
434 the rabbit. Reprod. Nutr. Dev. 29, 403-412.
435 Gidenne, T., Segura, M., Lapanouse, A., 2005. Effect of cereal sources and processing
436 in diets for the growing rabbit. I. Effects on digestion and fermentative activity in the
437 caecum. Anim. Res. 54, 55-64.
438 Hörnicke, H., 1981. Utilization of caecal digesta by caecotrophy (soft faeces ingestion)
439 in the rabbit. Livest. Prod. Sci. 8, 361-366.
440 Jouany, J.P., 1982. Volatile fatty acid and alcohol determination in digestive contents,
441 silage juices, bacterial cultures and anaerobic fermentor contents. Sci. Aliments 2,
442 131-144.
443 Leng, E., Hörnicke, H., 1976. Diurnal variations in the rabbits cecal volume *in vivo* with
444 C14-PEG. Zbl. Vet. Med. 23, 827-835.
445 Martín-Orúe, S.M., Balcells, J., Guada, J.A., Castrillo, C., 1995. Endogenous purine and
446 pyrimidine derivative excretion in pregnant sows. British Journal of Nutrition 73,
447 375-385.
448 Merino, J.M., Carabaño, R., 1992. Effect of type of fibre on ileal and fecal
449 digestibilities. J. Appl. Rabbit Res. 15, 931-937.
450 Nicodemus, N., García, J., Carabaño, R., De Blas, J.C., 2007. Effect of substitution of a
451 soybean hull and grape seed meal mixture for tradicional fiber sources on digestion
452 and performance of growing rabbits and lactating does. J. Anim. Sci. 85,181-187.
453 Nicodemus, N., Mateos, J., de Blas, J.C., Carabaño, R., Fraga, M.J., 1999. Effect of diet
454 on amino acid composition of soft faeces and the contrubution of soft faeces to total
455 amino acid intake through caecotrophy in lactating doe rabbits. Anim. Sci. 69, 167-
456 170.

457 Rooney, L.W., Pflugfelder, R.L., 1986. Factors affecting starch digestibility with special
458 emphasis on sorgum and corn. J. Anim. Sci. 63, 1607-1623.
459 SAS, 2004. Statistical Analysis System. SAS software package, version 9.1. SAS
460 Institute Inc., Cary, NC, USA.
461 Storm, E., Brown, D.S., Orskov, E.R., 1983. The nutritive value of rumen micro-
462 organism in ruminants. 3. The digestion of microbial amino and nucleic acids in, and
463 losses of endogenous nitrogen from the small intestine of sheep. Br. J. Nutr. 50, 479-
464 485.
465 Van Soest, P.J., Robertson, J.B., Lewis, R.A., 1991. Methods for dietary fiber, neutral
466 detergent fiber and non starch polysacharides in relation to animal nutrition. J. Dairy.
467 Sci. 74, 3583-3597.
468 Villamide, M.J., Carabaño, R., Maertens, L., Pascual, J., Gidenne, T., Falcao-E-Cunha,
469 L., Xiccato, G., 2009. Prediction of the nutritional value of European compound
470 feeds for rabbits by chemical components and *in vitro* analysis. Anim. Feed Sci.
471 Technol. 150, 283–294.
472 Villamide, M.J., Nicodemus, N., Fraga, M.J., Carabaño, R., 2010. Protein Digestion, in:
473 De Blas, C., Wiseman, J. (Eds.), The Nutrition of the Rabbit, 2nd ed., CAB
474 International, Wallingford, UK, pp. 39–55.
475 Xicatto, G., Trocino, A., 2010. Energy and protein metabolism and requirements, in: de
476 Blas, J.C., Wiseman, J. (Eds.). The Nutrition of the Rabbit, 2nd ed., CAB
477 International, Wallingford, UK, pp. 83-118.
478
479

480 **Table 1**. Ingredients (g/kg) and chemical composition (g/kg DM) of the experimental
481 diets formulated using two sources of fibre (alfalfa hay or sugar beet pulp) and two
482 sources of starch (maize or wheat), either labelled (Lab.; with $^{15}NH_4Cl$) or unlabelled
483 (Unlab.).

Source of fibre	Alfalfa hay				Sugar beet pulp			
Source of starch	Maize		Wheat		Maize		Wheat	
	Unlab.	Lab.	Unlab.	Lab.	Unlab.	Lab.	Unlab.	Lab.
Ingredients								
Sugar beet pulp	196	195	185	184	478	475	485	482
Alfalfa hay	506	502	516	512	159	158	162	161
Maize grain	184	183	0	0	149	148	0	0
Wheat grain	0	0	196	195	0	0	165	164
Soya bean meal	107	107	97	97	161	160	146	145
Treated straw	5	5	0	0	51	51	41	41
Sunflower oil	0	0	4	4	0	0	0	0
Vit-min mix†	2	2	2	2	2	2	2	2
Composition								
Dry matter (g/kg)	909	910	912	910	906	917	910	913
Organic matter	899	900	899	900	920	917	922	920
Digestible energy (MJ/kg) ‡	11.9	11.9	12.2	12.2	12.7	12.7	13.1	13.1
Crude protein	181	191	184	195	170	184	174	182
Digestible crude protein	130	137	141	149	132	143	136	143
NDF	338	336	324	333	350	342	341	356
ADF	176	180	181	182	182	184	181	184
Lignin	46	41	41	44	28	24	26	22

484 CP, crude protein (N x 6.25).

485 † Composition of vitamin-mineral mix: 200 ppm Co ($CoSO_4$ 7 H2O), 3000 ppm Cu ($CuSO_4$ 5 H2O),
486 20000 ppm Fe ($FeSO_4$ 1 H2O), 8000 ppm Mn (MnO_2), 30000 ppm Zn (ZnO), 30 ppm Se (Na_2SeO_3), 500
487 ppm I (KI), 4500000 IU/kg vit A, 550000 IU/kg vit D_3, 1100 ppm vit E, 250 ppm vit B_1, 1500 ppm vit B_2,
488 100 ppm vit B_6, 6000 ppm vit B_{12}, 500 ppm vit K, 5000 ppm D-pantothenate, 12500 ppm niacin, 100000
489 ppm choline chloride.

490 ‡ Estimated using the prediction equation: Digestible energy (MJ/kg) = 1.92 + 15.31 DM digestibility
491 (Villamide et al., 2009).

492

493 **Table 2.** Effect of dietary inclusion of alfalfa hay (AH) or sugar beet pulp (SBP), and
494 maize or wheat as sources of fibre and starch respectively on growth rate, dry matter
495 intake, digestible organic matter (OM) intake, and total tract apparent digestibility of
496 dry matter (DM), organic matter (OM), neutral detergent fibre (aNDFom) and nitrogen
497 (N), and physical and chemical characteristics of caecal contents (pH, NH_3 and volatile
498 fatty acid (VFA) concentrations, and proportions of acetic, propionic and butyric acids)
499 in growing rabbits.

	AH	SBP	Maize	Wheat	SE	Statistical significance	
						Fibre	Starch
Productive parameters							
Growth rate (g/d)	26.9	26.3	26.1	27.0	1.44	NS	NS
Intake (g/d):							
Dry matter	100.2	90.1	94.1	96.3	3.23	**	NS
Digestible OM	59.6	60.0	58.0	61.6	1.96	NS	NS
Digestible N	2.29	2.06	2.10	2.25	0.732	**	NS
% N recycled by caecotrophes	14.4	16.0	13.8	16.7	1.68	NS	NS
Digestibility:							
DM	0.66	0.71	0.68	0.70	0.010	***	*
OM	0.66	0.73	0.68	0.71	0.010	***	*
aNDFom	0.43	0.53	0.46	0.50	0.018	***	NS
N	0.74	0.78	0.75	0.77	0.021427	***	**
Caecum characteristics							
Physical:							
Full weight (g)	114	136	124	126	8.5	*	NS
Empty viscera (g)	32.9	37.1	35.0	35.0	1.16	**	NS
Chemical:							
pH	6.18	5.69	5.85	6.02	0.125	**	NS
NH_3 (mg/dL)	3.92	3.90	3.65	4.17	0.897	NS	NS
VFA (mmol/L)	67.5	89.6	82.3	74.8	7.54	**	NS
Acetic (mol/mol)	0.80	0.78	0.78	0.80	0.012	NS	NS
Propionic (mol/mol)	0.06	0.06	0.06	0.05	0.006	NS	NS
Butyric (mol/mol)	0.14	0.14	0.13	0.14	0.010	NS	NS

500 SE: Standard error of the treatment means; NS, non-significant; *, P<0.05; **, P<0.01; ***, P<0.001.

501

Table 3. Effect of dietary inclusion of alfalfa hay (AH) or sugar beet pulp (SBP), and maize or wheat as sources of fibre and starch, respectively, on the urinary excretion of purine derivatives (PD) and duodenal flow of purine bases (PB) in growing rabbits.

	AH	SBP	Maize	Wheat	SE	Statistical significance	
(μmol/W$^{0.75}$/d)						Fibre	Starch
PD excretion							
Alantoin	956	723	783	896	69.2	**	NS
Uric acid	79.5	57.6	70.1	67.0	7.73	**	NS
Total PD	1035	781	853	963	72.6	**	NS
Duodenal flow of PB							
Total†	1.545	1.165	1.273	1.438	0.1083	**	NS
Dietary	0.525	0.392	0.451	0.465	0.0129	***	NS
Microbial‡	1.066	0.808	0.861	1.013	0.1148	*	NS

SE: Standard error of the treatment means; NS, non-significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

† Estimated from urinary excretion of PD using the model proposed by Balcells et al. (1998), modified by Belenguer et al. (2008).

‡ Estimated by substraction of dietary PB ingested, assuming digestibility of 0.913 (Chen et al., 1990), from total duodenal flow of PB.

Table 4. Effect of dietary inclusion of alfalfa hay (AH) or sugar beet pulp (SBP), and maize or wheat as sources of fibre and starch, respectively, on ^{15}N-enrichments (atom % excess) in non-essential and essential amino acids in caecotrophes and liver in growing rabbits.

	Caecotrophes				Liver						Statistical significance			
	AH		SBP		AH		SBP		SE_1	SE_2	Fibre	Starch	Substrate	Fibre x Starch
Amino acids	Maize	Wheat	Maize	Wheat	Maize	Wheat	Maize	Wheat						
Non-essential														
Alanine	0.540	0.550	0.741	0.644	0.622	0.608	0.815	0.641	0.0190	0.0068	***	**	***	***
Glycine	0.411	0.389	0.597	0.488	0.467	0.434	0.630	0.516	0.0230	0.0162	***	**	NS	NS
Proline	0.165	0.187	0.239	0.236	0.118	0.115	0.182	0.136	0.0048	0.0051	***	NS	***	**
Serine	0.418	0.441	0.612	0.529	0.463	0.451	0.669	0.514	0.0154	0.0065	***	**	*	***
Aspartate	0.515	0.519	0.744	0.638	0.501	0.499	0.679	0.545	0.0137	0.0061	***	***	***	***
Glutamate	0.598	0.590	0.822	0.695	0.581	0.560	0.773	0.605	0.0171	0.0078	***	***	**	***
Tyrosine	0.446	0.459	0.565	0.528	0.202	0.197	0.303	0.205	0.0137	0.0101	***	*	***	*
Essential														
Valine	0.494	0.497	0.678	0.589	0.190	0.186	0.290	0.232	0.0125	0.0061	***	*	***	**
Leucine	0.502	0.505	0.692	0.594	0.202	0.199	0.312	0.238	0.0118	0.0061	***	**	***	***
Threonine	0.401	0.426	0.551	0.495	0.151	0.137	0.237	0.167	0.0103	0.0056	***	*	***	**
Phenylalanine	0.425	0.454	0.570	0.551	0.153	0.152	0.232	0.179	0.0122	0.0056	***	NS	***	**
Lysine	0.402	0.406	0.562	0.502	0.149	0.144	0.221	0.177	0.0100	0.0048	***	*	***	**

SE_1 and SE_2: standard errors of the animal effect and residual term in split-plot design; * NS, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 5. Effect of dietary inclusion of alfalfa hay (AH) or sugar beet pulp (SBP), and maize or wheat as sources of fibre and starch, respectively, on caecotrophe dry matter (DM) and N production and microbial nitrogen (MN) recycling using the following methods (M): caecotrophy prevention by using neck collar (CC), urinary excretion of purine derivatives (PD), or microbial ^{15}N-lysine incorporation (^{15}N-lysine) in growing rabbits.

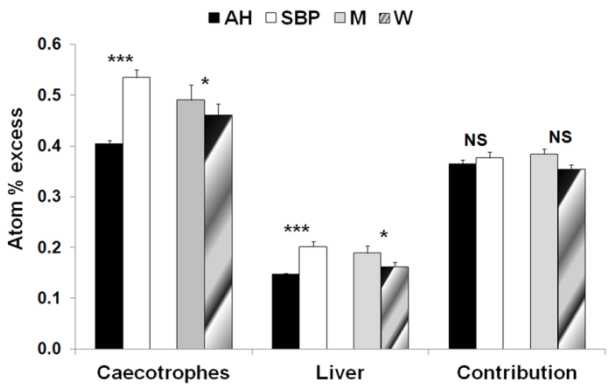
	AH	SBP	M	W	SE		Significance			
							Fibre	Starch		
Caecotrophes										
DM (g/d)	13.4	12.0	13.4	11.9	1.42		NS	NS		
N (g/d)	0.73	0.63	0.64	0.71	0.075		NS	NS		
	AH	SBP	M	W	SE_1	SE_2	Significance			
							Fibre	Starch	Method	Starch x M
MN recycling (g/d)					0.079	0.119	NS	NS	***	NS
CC	0.52	0.48	0.44	0.56						
PD	1.35	1.09	1.07	1.37						
^{15}N-lysine	1.37	1.39	1.46	1.30						

SE: Standard error of the treatment means; SE_1 and SE_2: standard errors of the animal effect and residual term in split-plot design; M, methodology; NS, non-significant; ***, $P < 0.001$.

Figure 1. ^{15}N-lysine enrichments (atom % excess) in caecotrophes and liver, and contribution of caecotrophes to tissue lysine in growing rabbits fed diets formulated based on alfalfa hay (AH) or sugar beet pulp (SBP) as main source of fibre, and those receiving diets with the inclusion of maize (M) or wheat (W) as source of starch (NS, non-significant; *, $P < 0.05$; ***, $P < 0.001$).

535

536


AH
SBP
M
W
Atom % excess
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
*
***
*
NS
NS
Caecotrophes
Liver
Contribution