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ABSTRACT

Protein turnover has been associated to residual feed intake (**RFI**) in beef cattle. However, this relationship may be confounded by feeding level and affected by the composition of the diet being fed. Our aim was to assess postmortem the protein metabolism signalling pathways in skeletal muscle and liver of 32 Charolais young bulls with extreme RFI phenotypes. Bulls were fed two contrasting diets during the whole fattening period but were subjected to a similar and single nutritional stimulus, induced by their respective concentrate, just prior to slaughter. The key targets were protein degradation (autophagy and ubiquitin) and synthesis signalling pathways through western-blot analysis, as well as hepatic transaminase activity. To ensure a precise assessment of all animals at the same postprandial time, they were provided with a test meal (2.5 kg of either a high-starch and high-protein concentrate or high-fibre and low-protein concentrate) 3 hours prior to slaughter, irrespective of their RFI grouping. Blood and tissues were sampled at the slaughterhouse (3 h and 3 h30 postprandially, respectively). In response to an identical single meal size, efficient RFI animals showed higher ($P < 0.05$) postprandial plasma β-hydroxybutyrate concentrations and insulinemia (only with the high-starch concentrate) than non-efficient animals. Moreover, efficient RFI bulls had lower muscle ($P = 0.04$) and liver ($P = 0.08$) ubiquitin protein abundance (degradation pathway) and tended to have lower alanine transaminase activity in the liver ($P = 0.06$) compared to non-efficient bulls, regardless of diet. A positive correlation between protein degradation potential and amino acid catabolism was identified in this study ($r = 0.52$, $P = 0.004$), which was interpreted as being biologically linked to the RFI phenotype. Efficient RFI bulls also had a faster potential for protein synthesis in the muscle, as indicated by their greater ratio of phosphorylated to total form of ribosomal protein S6 kinase ($P = 0.05$), regardless of diet. Results on protein synthesis pathway in muscle and plasma metabolite concentrations suggested that efficient RFI cattle may have a faster nutrient absorption and insulin responsiveness after feeding than inefficient cattle. We did not find significant differences in hepatic protein synthesis pathways between the two RFI groups ($P > 0.05$). Our findings suggest that, in response to an identical single meal size, efficient RFI animals exhibited lower activation of tissue protein degradation pathways and faster muscle protein synthesis activation compared to their inefficient counterparts. This pattern was observed regardless of the composition of the tested meals.

Implications

The link between animal feed efficiency and protein metabolism could be explained by differences in nutrient intake. To mitigate confounding factors, we evaluated the postprandial tissue protein metabolism in response to an identical single meal size in young bulls of contrasting residual feed intake and fattened on different diets. More efficient animals had lower tissue protein turnover, as evidenced by decreased muscle and liver protein degradation and lower hepatic transaminase activity, compared to less efficient animals. Although we only explored phenotypic correlations, these results suggest that selecting cattle for superior feed efficiency may also involve co-selection for lower protein turnover.

Introduction

Residual feed intake (**RFI**) is one of the preferred criteria for improving feed efficiency through genetic selection. This is because

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RFI has moderate heritability (Taussat et al., 2019) and selecting for it can decrease feed-related costs (Herd et al., 2003) without negatively affecting other performance traits of the reproductive herd like the mature BW of breeding cows (Johnson et al., 2003). However, selecting animals for RFI may have some drawbacks. First, RFI ranking of beef cattle is not repeatable when diet changes (Asher et al., 2018; Coyle et al., 2017; Lahart et al., 2020). This raises concerns about the reliability of RFI-based selection when animals are fed differently. Second, RFI selection may have unintended effects on other important animal traits, such as immunity or reproductive performances (Wang et al., 2012; Zerjal et al., 2021), as all these traits involve various energy-demanding mechanisms that can compete with each other when energy availability is limited. Balanced breeding programmes and avoidance of potential negative effects require a deeper understanding of the mechanisms behind RFI (Cantalapiedra-Hijar et al., 2018).

Continuous protein synthesis and degradation rates (referred to as protein turnover) are associated with high energy expenditure and have been suggested as key determinants of RFI in beef cattle (Richardson and Herd, 2004; Cantalapiedra-Hijar et al., 2018). Protein turnover could potentially be a key determinant of the trade-offs between production efficiency and robustness traits in livestock (Rauw, 2012). We confirmed previously in beef cattle that lower protein turnover in skeletal muscle, as indicated by *in vivo* fractional protein degradation rate, was associated with the efficient RFI phenotype (Guarnido-Lopez et al., 2022). However, this finding was observed when animals were fed high-starch diets, but not high-fibre diets, possibly due to differences in the amount and profile of absorbed nutrients (mainly glucogenic vs ketogenic) and the resulting insulin response.

The main challenge in evaluating potential biological mechanisms underlying RFI, such as protein turnover, is to determine whether the correlation is due to a covariation with feed (nutrients) intake level or a true cause-and-effect relationship (Cantalapiedra-Hijar et al., 2018). Due to the strong influence of nutrient intake level on metabolism (Reeds and Fuller, 1983; Boisclair et al., 1994; Lobley, 1998), it is challenging to determine whether the association between a specific mechanism and the RFI phenotype is due to differences in feeding level and appetite (Lines et al., 2014), or is related to host-related factors regardless of nutrient intake (Nkrumah et al., 2006). The molecular pathways involved in tissue protein metabolism exhibit high sensitivity to short-term and acute effects of nutrient intake, particularly the rise in glucose and amino acid absorption, as well as the subsequent insulin response. This sensitivity holds true for both monogastric (Norton et al., 2009) and ruminants (Mann et al., 2016). We propose that the association between RFI and tissue protein metabolism primarily arises from variations in the quantity of absorbed insulinogenic nutrients and amino acids, which serve as the primary drivers of protein synthesis signalling pathways (Laplante and Sabatini, 2009). Our specific hypotheses are as follows: (i) the signalling pathways related to protein metabolism will exhibit similarities between efficient and non-efficient RFI cattle when they are fed the same diet and exposed to an identical nutritional stimulus (i.e. an identical and single meal size) and (ii) the differences observed in the correlation between RFI and protein metabolism across diets (Guarnido-Lopez et al., 2022) will diminish when employing a uniform and consistent meal size before assessment.

Hence, the objective of this study was to determine if specific proteins involved in the regulation of the protein metabolism in cattle tissues were activated differently in efficient and inefficient RFI cattle. To evaluate tissue metabolic responses in animals subjected to an identical nutritional stimulus, all animals (including both low and high RFI) were fed a single and identical meal size consisting exclusively of the concentrate part of their diet, 3 h before slaughter. The tissues under focus are the skeletal muscle, which is the main body protein pool, and the liver, which is the major site for amino acid catabolism. We targeted the signalling pathways of protein degradation (adenosine monophosphate-activated protein kinase [**AMPK**], autophagy and ubiquitin pathways) and synthesis (serine/threonine protein kinase [**Akt**], mammalian target of rapamycin [**mTOR**], ribosomal protein S6 kinase [**S6K**] cascade) and the main hepatic transaminases related to amino acid catabolism.

Material and methods

The experiment was conducted at INRAE, Centre Auvergne-Rhône-Alpes, France. The experimental protocol was approved by the Ethics Committee of the Auvergne-Rhône-Alpes region and the French Ministry of Higher Education, Research and Innovation (Authorisation number: APAFIS #16194-2016101016361277 v6 delivered on the 14th of January 2019).

Animals, dietary management and experimental design

The present work consisted of a postmortem study that followed an *in vivo* trial (Guarnido-Lopez et al., 2022), which used a total of 32 extreme RFI fattening young bulls (561 ± 49.1 kg of BW; 1.24 ± 0.21 kg of average daily gain [**ADG**]; 9.06 ± 0.93 kg/d of DM intake; with a difference of DM intake between extreme RFI individuals = 1.10 kg/d). Half of the bulls were fed a high-starch corn-silage plus cereal-based diet, while the other half received a high-fibre grass-silage plus beet pulp-based diet. Both diets had a forage-to-concentrate ratio close to 60:40. Following an initial 84-d feed efficiency test conducted over 100 Charolais bulls in two consecutive years (2019 [n = 49] and 2020 [n = 51]), we selected the eight most extreme RFI individuals (the four lowest and highest RFI cattle) within each diet and experimental year for the *in vivo* analysis of protein metabolism and N partitioning using indirect methods. All 32 extreme RFI animals were slaughtered at the INRAE experimental abattoir (Saint Genès de Champanelle, France) at the end of the *in vivo* study.

In the present postmortem study, we assessed protein metabolism pathways in the skeletal muscle and the liver from the 32 extreme RFI individuals immediately after their slaughter (16 animals from 2019 and 16 from 2020). Slaughter took place over the course of one month (June 2019 and June 2020), with one animal from each diet and RFI class (low and high RFI) being slaughtered daily. Altogether four animals were slaughtered per week on the same day. This approach ensured that the average experimental duration (from the start to the slaughter) remained similar across treatments. Animals were fed their respective total mixed ration for approximately 200 d prior to the day of slaughter (Guarnido-Lopez et al. 2022).

Animals underwent a fasting period beginning 15 hours prior to slaughter, followed by a single test meal containing an equivalent quantity of their respective concentrate (2.5 kg as fed) and fed 3 h prior to slaughter. Because of their nature, these concentrates presented a more contrasted nutritional composition (high-starch and high-protein concentrate or high-fibre and low-protein concentrate; Table 1) compared to their corresponding total mixed ration (Guarnido-Lopez et al., 2022). We checked that all animals completely consumed their meal. The rationale is as follows. During the postprandial period, circulating insulin and subsequent amino acid uptake in the skeletal muscle tissue increase. The postprandial period represents the physiological window during which intramuscular anabolic signalling is stimulated (Cuthbertson et al., 2005). Previous exploratory analyses of protein turnover rates in beef cattle (Cantalapiedra-Hijar et al., 2020) indicated that the intensity of protein synthesis signalling pathways was rather low

Table 1
Dietary characteristics of experimental concentrates given to young Charolais bulls 3 h before the slaughter.

Item	High-fibre and low-protein concentrate	High-starch and high-protein concentrate
Ingredient composition, kg DM		
Wheat grain	0.50	1.48
Beet pulp	1.62	–
Soybean meal	0.37	0.99
Bicarbonate	–	0.20
Minerals and vitamin mix*	0.10	0.10
Chemical composition (g/kg of DM)		
OM	91.7 ± 0.37	93.2 ± 0.22
CP	167 ± 0.38	289 ± 1.41
NDF	311 ± 1.31	130 ± 3.15
ADF	160 ± 1.10	52.9 ± 0.21
Starch	131 ± 0.69	374 ± 2.07
Starch/NDF (g/g)	0.42 ± 0.00	2.96 ± 0.87
In vitro enzymatic digestibility[1] (%)		
DM	92.1 ± 0.39	94.9 ± 2.12
Organic matter	92.9 ± 0.33	95.2 ± 1.20
Feed Values[2]		
Net Energy (Mcal/kg)	2.00 ± 0.00	2.22 ± 0.01
MP (g/kg of DM)	118 ± 2.71	153 ± 8.58
MP/Net energy (g/Mcal)	58.9 ± 0.20	68.9 ± 1.14
RPB (g/kg of DM)[3]	−3.87 ± 1.80	84.1 ± 4.95

Abbreviations: MP = metabolisable protein; RPB = rumen protein balance.
* Minerals and vitamin mix: 5% P, 25% Ca, 8% Mg, 0·2 % Na, vitamin A (210 mg/kg), vitamin D3 (3.75 mg/kg), vitamin E (2 730 mg/kg) and vitamin B1 (4.5 mg/kg).
[1] Estimated from enzymatic digestion with pepsin-cellulase (Aufrère, 1982).
[2] Feed values were estimated from chemical composition and proportion of ingredients (INRA, 2018).
[3] Rumen protein balance, representing nitrogen intake minus non-ammonia nitrogen at the duodenum (INRA, 2018).

when assessed in a non-stimulated condition, i.e. in a fasting state (Cantalapiedra-Hijar G., personal observation). In this regard, glucose intravenous infusion in dairy cows resulted in a rise in insulinemia, which was sufficient to activate within 1 h the mTOR pathway in the muscle of dairy cows (Mann et al., 2016). Based on these findings, we decided to conduct the postmortem study with animals in the postprandial state, following a single meal size administered after a 12-hour food deprivation period. This methodology has been utilised in studies involving rats, piglets and horses (Norton et al., 2009; Gazzaneo et al., 2011; Loos et al., 2020). Because the explored signalling pathways respond to a meal stimulus, it was important to set all individuals at the same postprandial time. The purpose of the short food deprivation period was to stimulate a prompt and complete consumption of the provided meal while minimising differences between individuals due to non-synchronised nutritional conditions (i.e. meal consumption at different times). By offering only the concentrate as the meal, we aimed to minimise any discrepancies in both the duration and quantity of feed consumed among the animals that would likely be observed if the total mixed ration had been used instead. This protocol was designed to guarantee that key proteins involved in the regulation of the protein metabolism in cattle tissues would be assessed in the same nutritional conditions for all animals fed the same diet. Finally, we chose a 3 hours interval between the last meal and slaughter because it corresponds to the postprandial absorption peak of glucose and insulin in cattle (Jenny and Polan, 1974; Evans et al., 1975), both substrates acting as signals for protein synthesis and degradation (Reeds and Fuller, 1983). All animals were slaughtered strictly 3 hours after the distribution of their respective test meal.

Measurements and sampling

Plasma and tissue sampling

Blood samples were collected at slaughter (i.e. 3 h after the test meal) by jugular venipuncture once the animal was hung. Blood samples were collected into a 9 ml EDTA tube (BD vacutainer, Plymouth, UK) and centrifuged at 2 500*g* for 10 min at room temperature to obtain the plasma, which was subsequently stored at −20 °C until the analysis of metabolites (glucose, urea and β-hydroxybutyrate) and hormones (insulin and IGF-1) concentrations.

Approximately 30 min after the slaughter (i.e. 3 h and 30 min after the distribution of the test meal), both hepatic (caudal lobe) and muscle (*longissimus dorsi*) tissues were sampled in order to evaluate the postprandial protein synthesis and degradation intracellular pathways, as well as the activity level of key enzymes involved in amino acid metabolism. Tissue samples were quickly cut into small squares of 1 cm × 1 cm, frozen immediately in liquid nitrogen and stored at −80 °C awaiting analyses.

Laboratory analyses

Concentrates were analysed for DM, organic matter (**OM**), CP, NDF, ADF and starch, and their feeding values were calculated as described in Guarnido-Lopez et al. (2022). Chemical composition and feeding values are reported in Table 1.

Plasma metabolites and hormone analyses

For plasma glucose, β-hydroxybutyrate and urea concentrations, spectrophotometric analyses were conducted using an automated analyzer (Arene 20XT, Thermo Scientific, Vaanta, Finland). Urea analyses were conducted in duplicate, whereas glucose and β-hydroxybutyrate analyses were performed as single measurements. The accuracy profile (NF V03-110: 2010) of the automated analyser for the concentration of these three metabolites ranged between 0.05 and 0.90 g/L, yielded an average accuracy of 101% and a CV for replicates averaging 8%. Plasma insulin and IGF-1 concentrations were determined by radioimmunoassay (RIA) as detailed in Jorge-Smeding et al. (2021).

Molecular analyses

The relative abundance of proteins involved in protein synthesis and degradation were quantified in hepatic and muscle samples through western blot. Frozen samples were homogenised on ice

in 2 ml lysis buffer (150 mM NaCl, 10 mM Tris, 1 mM ethylene glycol tetraacetic acid, 1 mM ethylenediaminetetraacetic acid [**EDTA**], pH 7.4, 100 mM Sodium Fluoride, 4 mM Sodium pyrophosphate, 2 mM Sodium Orthovanadate, 1% Triton X100, Protease inhibitor cocktail to 1% V/V) before using (Merck Sigma, P8340 Saint Quentin Fallavier). The homogenate obtained was centrifuged at 10 000*g* for 15 min at 4 °C. Protein concentration was determined using a Bicinchoninic acid assay protein assay kit (Thermo Pierce Biotechnology, IL, USA) according to the protocol of the manufacturer with bovine serum albumin (**BSA**) as a standard. Proteins were then denaturised in a 1X Laemmli solution (+β-mercaptoethanol) at 95 °C for 5 min, and separated onto polyacrylamide gels (Biorad, Mini-Protean TGX stain-Free gels, 4–20%, 4568095, Mitry-Mory). Equal amounts of protein (20 µg per well) were loaded into gels set on a Mini-Protean tetra electrophoretic system (Bio-Rad) and run in a 1X buffer (Running buffer 10X, Tris-Base, Glycine and SDS) for 2 h at 80 V at ambient temperature and 70 V until the blue reached the bottom of the gel. After migration, separated proteins were transferred onto polyvinylidene fluoride membranes (Biorad, Trans-Blot Turbo, transfer kit, LF PVDF, 1704275). After washing in a phosphate-buffered saline-Tween 1X solution, membranes were blocked for 1 h at room temperature in blocking solution, BSA 5% PBS-Tween 0.1%.

A total of eight samples were disposed by gel balanced for the experimental effects (RFI group, diet type and the experimental period [2019 and 2020]). In addition, two standard samples of each tested protein were included in every gel. For protein synthesis, we analysed the Akt, mTOR and S6K pathways. Antibodies utilised for Akt pathway were Akt (pan, rabbit ab #4691, Cell Signalling, Ozyme, France) and pAkt (Serine 473, rabbit Ab #4060, Cell Signalling, Ozyme, France). Antibodies utilised for mTOR pathway were mTOR (pan, rabbit ab #2972, Cell Signalling, Ozyme, France) and pmTOR (Serine 2448, rabbit Ab #39182, Cell Signalling, Ozyme, France). Antibodies utilised for S6K pathway were S6K (S6 ribosomal protein #2217 Cell Signalling, Ozyme, France) and pS6K (Serine 235/236, rabbit Ab #2211, Cell Signalling, Ozyme, France). For protein degradation (proteolysis), we analysed the AMPK, ubiquitin-like modifier activating enzyme 1 (**UBA1**) and the microtubule-associated protein 1A/1B-light chain 3 (**LC3**) pathways. Antibody utilised for UBA pathway was UBA1 (Anti-E1 Ubiquitin Activating Enzyme 1, ab228793, Abcam, Amsterdam). Antibody used for LC3 pathway was LC3B (Anti-LC3B antibody produced in rabbit, ~1 mg/mL, L7543, Sigma Aldrich-Merck, France). For AMPk, we utilised AMPk (AMPKalpha #2532, Celle Signalling, Ozyme, France) and pAMPk (Phospho-AMPKalpha (Thr 172) #2531, Cell Signalling, Ozyme, France).

Membranes were incubated overnight at 4 °C, and protein bands were visualised by Infrared Fluorescence using the Odyssey Imaging System (LI-COR Inc. Biotechnology, Lincoln, NE, USA) and quantified by Odyssey infrared imaging system software (Application software, version 1.2). Finally, we normalised intracellular results of the activation of both protein synthesis and degradation according to the total quantity of proteins in these pools.

Hepatic enzyme activities

Enzyme activities of the two main hepatic transaminases, alanine amino-transferase (**ALT**) and aspartate amino-transferase (**AST**) were evaluated. Samples were homogenised with ice-cold buffer (10 w:v; 50 mM Tris, pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM Sodium orthovanadate, 5 mM Sodium pyrophosphate, 50 mM NaF, 1 mM EDTA, 1% V/V Triton X100, 1% V/V Protease Inhibitor Cocktail). The homogenate was centrifuged at 10 000*g* for 15 min at 4 °C and the supernatant was used immediately for enzyme assays. Analyses were conducted through spectrophotometry using a microplate reader (Infinite® 200 PRO NanoQuant, Tecan, Grödig, Austria) as described in Polakof et al., (2008) and adapted for protein concentrations in cattle tissues through a previous set-up.

Statistical analysis

Statistical analyses were performed in R (RStudio Core Team, version 1.1.463, 2018). To test the effects of the diet, the RFI group and their interaction on all postmortem analysed variables, a general linear model was run that included the year, the nature of the diet, the RFI group, and their interactions as fixed effects (Experimental year, Diet, RFI, Year × Diet, Year × RFI and Diet × RFI);

$$Y_{ijkl} = \mu + D_i + A_k + E_j + D_i \times A_k + E_i \times A_k + D_i \times E_j + {}_{ijkl}.$$

where Y_{ijkl} is the dependent variable for animal i, belonging to the RFI group l, receiving diet j, in year k, µ is the overall mean; Dj is the fixed effect of diet used (j = high-starch and high-fibre concentrate), A_k is the fixed effect of the year (k = 2019 and 2020), El is the fixed effect of the RFI group (l = efficient and non-efficient), Dj × Ak is the interaction between the effect of the diet and the year, El × A_k is the interaction between the effect of the year and the RFI group, Dj × El is the interaction between the effect of the diet and the RFI group and the ε_{ijkl} is the residual term. The triple interaction and the week in which the animals were slaughtered were also tested. However, since they did not show significance across all variables, they were not incorporated into the model. Finally, and just in the case of western-blot analyses, we added the effect of the agar gel number (n = 4 and 16 for protein degradation and synthesis proteins, respectively) as a fixed effect.

Mean values are reported with associated errors (SEM). When the Diet × RFI interaction was significant, the means for the different RFI groups were compared using Tukey's significant difference multiple comparison. Effects were declared significant when $P \leq 0.05$, and a trend was considered when $0.05 < P < 0.10$.

Results

All variables were assessed for normality and followed a normal distribution ($P > 0.05$). Interactions between year, RFI group and diet type were tested, and they were non-significant and are not presented in the tables ($P > 0.05$). Furthermore, the statistical effect of the gel number on western-blot results was not significant ($P > 0.05$). We have decided not to present the analysis of hepatic mTOR and AMPK proteins due to poor image quality and intensity of bands.

Plasma metabolites and hormone concentrations

Regardless of the composition of the test meal, efficient RFI animals had higher plasma β-hydroxybutyrate concentrations (+28.6%; $P = 0.03$) than non-efficient animals (Table 2). In contrast and only when fed a high-starch concentrate as the test meal, animals with efficient RFI showed higher insulinemia (+35.2%, Diet × RFI interaction; $P = 0.03$) and a tendency towards higher glycaemia (+16.7%, Diet × RFI interaction; $P = 0.08$) compared to animals with non-efficient RFI. There were no differences in plasma urea or IGF-1 levels across the tested concentrates or RFI groups ($P \geq 0.13$). With regards to the diet effect, animals fed a high-starch concentrate had higher plasma insulin concentrations (+21%, $P = 0.05$) and a lower glucose-to-insulin ratio (−35.3%, $P = 0.01$) compared to animals fed a high-fibre concentrate.

Table 2
Postprandial plasma metabolites and hormones concentrations from extreme residual feed intake (RFI) young Charolais bulls fed an identical single meal size of either a high-fibre and low-protein concentrate or high-starch and high-protein concentrate 3 h before slaughter.

Diet	High-fibre and low-protein concentrate		High-starch and high-protein concentrate			*P*-value		
RFI group	Low RFI	High RFI	Low RFI	High RFI	SEM	Diet	RFI	Diet × RFI
Number of animals	8	8	8	8				
Metabolites								
Glucose, g/L	0.61	0.65	0.66	0.55	0.039	0.64	0.53	0.08
BHB, mmol/L	0.31	0.25	0.32	0.26	0.022	0.52	0.03	0.76
Urea, g/L	0.17	0.16	0.19	0.18	0.014	0.13	0.43	0.93
Hormones								
IGF-1, ng/mL	334	301	363	354	31.9	0.18	0.57	0.77
Insulin, μU/mL	11.0[b]	17.5[b]	25.6[a]	16.6[b]	3.09	0.05	0.58	0.03
Glucose to insulin ratio, g/μU	0.55	0.48	0.32	0.35	0.013	0.01	0.75	0.43

Abbreviations: BHB = β-hydroxybutyrate.
[a,b] Values with different letters on the same row are significantly different ($P < 0.05$).

Table 3
Relative abundances of main proteins involved in the skeletal muscle protein degradation and synthesis pathways from extreme residual feed intake (RFI) young Charolais bulls fed an identical single meal size of either a high-fibre and low-protein concentrate or high-starch and high-protein concentrate 3 h before slaughter.

Diet	High-fibre and low-protein concentrate		High-starch and high-protein concentrate			*P*-value		
RFI group	Low RFI	High RFI	Low RFI	High RFI	SEM	Diet	RFI	Diet × RFI
Number of animals	8	8	8	8				
Protein degradation								
AMPK	1.00	0.94	1.04	1.00	0.051	0.33	0.30	0.88
LC3	1.03	1.00	0.98	1.02	0.061	0.83	0.91	0.62
UBA1	0.79	1.31	0.87	1.12	0.173	0.74	0.04	0.47
Protein synthesis								
Akt	1.02	1.11	0.84	1.00	0.060	0.06	0.05	0.65
p-Akt	0.95	1.20	0.87	0.96	0.095	0.12	0.11	0.44
p-Akt:Akt	0.94	1.06	1.02	0.96	0.081	0.87	0.76	0.26
mTOR	0.92	1.17	0.86	0.97	0.124	0.25	0.10	0.54
p-mTOR	0.93	1.26	0.74	1.21	0.159	0.23	0.01	0.39
p-mTOR:mTOR	1.13	1.15	0.76	1.28	0.198	0.46	0.10	0.09
S6K	0.81	1.03	0.87	1.35	0.205	0.31	0.07	0.50
p-S6K	0.79	0.89	1.14	1.25	0.233	0.14	0.67	0.98
p-S6K:S6K	1.18	1.01	1.48	0.88	0.187	0.64	0.05	0.26

Abbreviations: AMPK = Adenosine monophosphate-activated protein kinase; LC3 = Microtubule-associated protein 1A/1B-light chain 3; UBA1 = Ubiquitin-like modifier activating enzyme 1; Akt = Serine/threonine protein kinase; p-Akt = Phosphorylated serine/threonine protein kinase; mTOR = Mammalian target of rapamycin; pmTOR = Phosphorylated mammalian target of rapamycin; S6K = Ribosomal protein S6 kinase; pS6K = Phosphorylated ribosomal protein S6 kinase.

Signalling pathways related to muscle protein degradation and synthesis

Efficient RFI bulls had a lower potential for intracellular protein degradation in the muscle, as evidenced by their lower (−34.6%, $P = 0.04$) ubiquitin relative abundance (UBA1) compared to non-efficient bulls, regardless the composition of the test meal (Table 3). There were no differences in autophagy (LC3) or AMPK signalling pathways between the two RFI groups ($P \geq 0.30$). With regards to protein synthesis, efficient RFI bulls had a lower abundance of total Akt (−24.4%, $P = 0.05$) and S6K (−26.8%, $P = 0.07$) proteins, but no changes were observed in their respective phosphorylated forms ($P \geq 0.11$). In contrast, phosphorylated mTOR protein abundance was higher (+35%, $P = 0.01$) in non-efficient bulls regardless of the composition of the test meal, and a similar trend was observed for total mTOR abundance (+24.3%, $P = 0.10$). Overall, efficient RFI individuals presented a higher potential for protein synthesis in the muscle regardless of the composition of the test meal, as supported by their higher (+30.2%, $P = 0.05$) pS6K to S6K ratio. However, the pmTOR to mTOR ratio was higher in non-efficient RFI bulls when fed high-starch concentrate (+40.1%, Interaction RFI × Diet; $P = 0.09$). There were no diet effects observed for the analysed protein signalling pathways in the muscle ($P > 0.05$).

Hepatic amino acid metabolism and signalling pathways related to protein degradation and synthesis

As shown in Table 4, efficient RFI bulls had a trend for lower hepatic ALT activity (−14.2%, $P = 0.06$) compared to non-efficient bulls. We did not find any significant differences in hepatic protein synthesis (Akt and S6K) or protein degradation (LC3 and UBA1) pathways between the two RFI groups ($P \geq 0.08$). With regards to the effects of the composition of the test meal, animals fed a high-starch concentrate had higher protein degradation potential, as indicated by UBA1 abundance (+52%, $P = 0.001$). Furthermore, only efficient RFI bulls showed a higher relative abundance of pAkt (+19.1%, Diet × RFI interaction; $P = 0.04$) and a trend for a higher pAkt to Akt ratio (+23.3%, Diet × RFI interaction; $P = 0.09$) compared to inefficient bulls when fed the high-starch concentrate.

Discussion

We previously reported, using different approaches, greater nitrogen use efficiency in efficient RFI beef cattle compared with their inefficient counterparts whatever the diet (Cantalapiedra-Hijar et al., 2020; Guarnido-Lopez et al., 2021; Guarnido-Lopez

Table 4
Relative abundances of main proteins involved in the liver protein degradation and synthesis pathways and hepatic transaminase activity from extreme residual feed intake (RFI) young Charolais bulls fed an identical single meal size of either a high-fibre and low-protein concentrate or high-starch and high-protein concentrate 3 h before slaughter.

Diet	High-fibre and low-protein concentrate		High-starch and high-protein concentrate			*P*-value		
RFI group	Low RFI	High RFI	Low RFI	High RFI	SEM	Diet	RFI	Diet × RFI
Number of animals	8	8	8	8				
Protein degradation*								
LC3	1.01	0.97	0.99	1.02	0.049	0.80	0.99	0.51
UBA1	0.66	0.70	1.19	1.44	0.081	0.001	0.08	0.19
Protein synthesis*								
Akt	0.96	1.02	0.99	1.03	0.039	0.47	0.16	0.64
p-Akt	0.90[c]	1.05[b]	1.11[a]	0.91[c]	0.078	0.69	0.72	0.04
p-Akt:Akt	0.94	1.03	1.16	0.89	0.099	0.78	0.33	0.09
S6K	1.04	0.96	1.03	0.90	0.057	0.57	0.08	0.68
p-S6K	0.99	1.04	1.08	0.84	0.113	0.45	0.29	0.33
p-S6K:S6K	0.95	1.12	1.09	1.01	0.148	0.91	0.93	0.51
Enzymatic activity, mU/mg prot**								
AST	1 239	1 275	1 121	1 447	164.5	0.89	0.15	0.39
ALT	64.2	75.2	63.2	73.5	5.56	0.81	0.06	0.94

Abbreviations: LC3 = Microtubule-associated protein 1A/1B-light chain 3; UBA1 = Ubiquitin-like modifier activating enzyme 1; Akt = Serine/threonine protein kinase; pAkt = Phosphorylated serine/threonine protein kinase; S6K = Ribosomal protein S6 kinase; pS6K = Phosphorylated ribosomal protein S6 kinase; AST = Aspartate amino-transferase; ALT = Alanine amino-transferase; AMPK = Adenosine monophosphate-activated protein kinase; mTOR = Mammalian target of rapamycin.
* The results of AMPK and mTOR were not exploitable due to a weak expression of proteins in gels.
** Enzymatic activity was measured over thawed samples of liver from extreme RFI bulls. Units are given in milliunit (mU).
[a,b,c] Averages with different letters on the same row are significantly different ($P < 0.05$).

et al., 2022) in agreement with conclusions found in dairy cows (Liu and VandeHaar, 2020). We also found that efficient RFI beef cattle exhibit more protein deposition in the carcass at the expense of the organs only when animals were fed a high-starch (vs high-fibre) diet (Meale et al., 2017; Jorge-Smeding et al., 2021; Guarnido-Lopez et al., 2022). Protein turnover is considered to be a major determinant of RFI variations (Cantalapiedra-Hijar et al., 2018) and could contribute to explain the relationship between nitrogen use efficiency, body composition and RFI values previously reported. A positive association between protein turnover and RFI was observed, but this association was evident only in animals fed a high-starch diet and not in those on a high-fibre diet, which is also associated with higher insulinemia (Jorge-Smeding et al., 2021; Guarnido-Lopez et al., 2022). However, it remained uncertain whether this association was primarily attributed to differences in nutrient signalling among animals with extreme RFI.

In the present study, our main findings show that, in response to an identical meal size and independently of the composition of the test meal (concentrates), the efficient RFI cattle had lower protein degradation rates in muscle and liver, as well as lower hepatic transaminase activity than the inefficient RFI cattle whatever the diet. They also demonstrated a faster activation of protein synthesis in muscle. These results highlight the potential role of protein synthesis and degradation in contributing to differences in protein deposition that characterise individuals with extreme RFI phenotype.

Relationship between protein degradation, nitrogen use efficiency and residual feed intake

In addition to their related energy expenditures, lower protein degradation rates and amino acid catabolism could potentially contribute to an improved RFI by enhancing the nitrogen use efficiency (Cantalapiedra-Hijar et al., 2020). The lower activation of tissue protein degradation pathways and transaminase activity, as observed in our study following a similar nutritional stimulus, in efficient compared to non-efficient RFI individuals may explain the previously reported association between nitrogen use efficiency and RFI (Cantalapiedra-Hijar et al., 2020; Liu and VandeHaar, 2020; Guarnido-Lopez et al., 2022).

Lower protein degradation rates have been reported in muscle of efficient RFI cattle (McDonaugh et al., 2001) and pigs (Cruzen et al., 2013) fed ad-libitum compared to their non-efficient RFI counterparts. We analysed in the present study, the lysosomal (LC3) and the ubiquitin (UBA1) proteins as the two major markers of the intracellular protein degradation pathways. The lysosomal pathway plays a role in autophagy of long-lived proteins (Mizushima et al., 2008), while ubiquitin pathway degrades damaged, non-functional or misfolded proteins (Goldberg, 2003). We only found a significant difference in the ubiquitin pathway, which showed a positive correlation with RFI values ($r = 0.42$, $P = 0.02$), suggesting non-efficient RFI individuals may have a higher rate of degradation for damaged, misfolded or non-functional proteins in muscle. Indeed, the higher tissue protein degradation rate in non-efficient animals may result in increased post-transcriptional protein modifications (Bozaykut et al., 2014), such as N-terminal acetylation, which can in turn increase the creation of misfolded proteins and trigger the ubiquitin pathway, leading to further protein degradation (Hwang et al., 2010). The autophagy pathway was not found to be different, because it is mainly triggered by reduced ATP levels (Wang et al., 2013) likely not occurring in our postprandial conditions. In contrast to our findings, other studies have reported that genes associated with the autophagy pathway were downregulated in efficient RFI cattle (Rolf et al., 2012), but signalling information was lacking on those studies, limiting the functional conclusions to be drawn.

Results concerning the transaminase activity in the liver suggest lower amino acid catabolism in efficient RFI cattle. Both AST and ALT play a role in amino acid transamination, a necessary step for most amino acids to be catabolised (Torchinsky and Berezov, 1987). Differences in amino acid catabolism between extreme RFI cattle were also suggested by Cantalapiedra-Hijar et al. (2020), who observed lower transamination rates of individual plasma amino acids in efficient vs non-efficient RFI Charolais bulls using indirect isotopic methods. Based on the findings of Righetti et al. (1971), which reported that on average 80% of amino acids released from protein degradation can be recycled for protein synthesis, it can be inferred that approximately 20% of amino acids released during each round of protein degradation will be catabolised. Therefore, higher rates of protein degradation may result in greater amino acid catabolism, as observed in uremic human patients (Mitch et al., 1993) or in ruminants (Bequette, 2003). In the current study, we found a positive correlation ($r = 0.52$, $P = 0.004$) between the hepatic protein degradation potential

(UBA1 protein abundance) and the hepatic transaminase activity (ALT activity), irrespectively of the diet, but did not observe any correlations with targets involved in the protein synthesis pathway ($P > 0.27$).

Residual feed intake and tissue protein synthesis pathway

Although both groups were fed the same meal size 3 h before slaughter, efficient RFI cattle had higher postprandial plasma concentrations of β-hydroxybutyrate (both concentrates) and insulin (only for the high-starch–high-protein concentrate) compared to their inefficient counterparts. These metabolic features, along with the changes observed in the mTOR pathway, may suggest that the absorption of nutrients and subsequent activation of muscle protein synthesis occurred faster in efficient RFI animals in response to an identical single meal size. However, it is worth noting that no changes were observed in the hepatic protein synthesis pathways, likely because insulin does not stimulate protein synthesis in visceral tissues to the same extent as it does in skeletal muscle (Davis et al., 2001).

In our previous *in vivo* study (Guarnido-Lopez et al., 2022), we did not directly measure the skeletal muscle protein synthesis rate. However, based on the data on carcass muscle proportion and protein degradation rates, we estimated slightly lower muscle protein synthesis rates in efficient RFI animals that were fed a corn-based diet. We attributed this finding to the lower circulating insulin and branched-chain amino acids in these animals compared to inefficient RFI bulls, as previously reported by Jorge-Smeding et al. (2021) and Guarnido-Lopez et al. (2022). In our present postmortem study, we found that the plasma insulin levels in animals fed the high-starch concentrate were greater in efficient RFI individuals than in inefficient RFI individuals, despite both groups being fed an identical meal size prior to slaughter. This finding could be interpreted as efficient RFI animals had a greater insulin responsiveness to nutrients than inefficient ones. This is also coherent with studies performed in chicken (Gabarrou et al., 2000) and pigs (Colpoys et al., 2019), where RFI-efficient animals globally presented higher rises in plasma insulin levels following a glucose tolerance test. In contrast, the study by Fitzsimons et al. (2014) failed to evidence a significant difference in insulin responses and sensitivity across RFI groups following a glucose tolerance test. We did not observe the same finding when animals were fed the high-fibre concentrate, likely because the amount of glucose absorbed from the intestine with this kind of diet was not enough to elicit changes in insulin responsiveness (Jorge-Smeding et al., 2021).

In the present study, in response to an identical single meal size, efficient RFI individuals had greater pS6K to S6K ratio in muscle tissues compared to their inefficient counterpart, irrespectively of the composition of the test meal. Phosphorylation of protein S6 kinase is one of the last steps leading to increases in protein synthesis (Laplante and Sabatini, 2009) by stimulating messenger RNA translation (Kolitz and Lorsch, 2010). Thus, our results may be interpreted as a faster activation of protein synthesis for efficient RFI animals in response to an identical single meal size.

Considering the chronological sequence of events within the mTOR signalling cascade subsequent to its activation by insulin and amino acids (Seiliez et al., 2008; Wang et al., 2021), we can hypothesise that nutrient signalling on this pathway may have been faster in efficient RFI cattle. This hypothesis is compatible with the expected faster glucose and amino acid absorption rates when the same amount of concentrate should transit lower rumen sizes, anatomical feature found for these animals (Guarnido-Lopez et al., 2022). These factors could have led to faster insulin secretion and protein synthesis activation in efficient RFI animals. Insulin secretion in response to meal intake can be as fast as 5 minutes in growing cattle (Chase et al., 1976), leading to a peak in plasma insulin concentration between 1 and 4 hours after feeding, according to different experimental conditions in ruminants (Jenny and Polan, 1974; Evans et al., 1975; Vicari et al., 2008). In the same line, the greater β-hydroxybutyrate plasma concentrations in efficient RFI cattle, regardless of the composition of the concentrate, may also agree with a faster nutrient absorption rate in these animals. The portal and posthepatic absorption of β-hydroxybutyrate in animals with positive energy balance (as those used in this experiment) depends mostly on the amount of OM fermented in the rumen (Loncke et al., 2009; 2015) and is primarily derived from the partial oxidation of butyrate in the rumen wall (Noziere et al., 2000; Loncke et al., 2015). In this regard, based on the abundance of proteins involved in mTOR pathway and gene expression in the rumen epithelium, Elolimy et al. (2019) speculated that efficient RFI cattle could have an improved rumen absorption of nutrients such as butyrate.

The faster protein synthesis and lower protein degradation rates observed in our postmortem study in muscle of efficient RFI cattle, regardless of the nature of the concentrate, should logically have resulted in greater protein accretion rate. However, this finding was only observed *in vivo* in animals fed the corn-silage diet, and not in those fed the grass-silage diet (Guarnido-Lopez et al., 2022). This difference is likely because the absorption rate and nature of nutrients varied more significantly between the two total mixed rations used in vivo than across the experimental concentrates used in this experiment.

Finally, as suggested by Berry and Crowley (2013), there is evidence that RFI tends to favour the selection of later-maturing animals. All else being equal, animals of the same age that deposit proportionally more protein than fat for the same daily gain will, on average, be considered more efficient (low RFI). Considering the role of protein synthesis and degradation rates in protein retention, it is reasonable to anticipate that later-maturing animals (with low RFI) would display either lower protein degradation rates, higher protein synthesis rates, or a combination of both compared to earlier-maturing animals (with high RFI) of the same age.

Limitations of the study

The experimental design we employed in our study was inspired by previous research conducted in monogastric species, which focused on evaluating protein metabolism signalling pathways in response to an identical nutritional stimulus, namely a single and identical meal size, following a fasting period of 12 hours (Norton et al., 2009; Murgas Torrazza et al., 2010; Gazzaneo et al., 2011). In these studies, the single meal given to the animals before the evaluation differed in both quality and quantity from the previous meals with the basal diet. The rationale behind this approach lies in the understanding that the activation state of the signalling pathways related to protein synthesis in muscle is mainly triggered by the immediate stimulus of absorbed nutrients (glucogenic nutrients and amino acids) and the subsequent insulin response (O'Connor et al., 2003). Similar mechanisms are expected to occur in ruminants, as evidenced by the activation of the mTOR pathway in the muscle of dairy cows triggered as early as 1 hour after glucose intravenous infusion (Mann et al., 2016). Feeding all individuals in a similar manner before the assessment was intended to create a more comparable nutritional scenario for subsequent analysis.

Ruminants, however, differ from monogastric animals because of their particular digestive anatomy; their rate of feed digestion is much slower, and the profile and kinetics of nutrient absorption differ. As a result, the experimental model may present limitations and results should be interpreted with caution. Fasting for 12 hours in ruminants and distribution of a single and identical meal

size to all animals do not entirely eliminate the influence of previous meals and historic intakes on the nutrient absorption and metabolism, including the studied signalling pathways. While we observed differential activation of the evaluated pathways across extreme RFI groups, this could lead to the interpretation that the differences are independent of feed intake levels since all animals received the same meal size before evaluation. However, we cannot disregard the possibility of a minor effect from the previously observed contrasting feed intake across both RFI groups (Guarnido-Lopez et al., 2022) on the outcomes we have obtained in the current study. Indeed, long-term or chronic effects of enteral oil infusion on insulin signalling pathways (Akt-mTOR-S6K) were found in the muscle of dairy cows in a fed steady-state (Gingras et al., 2007). Also, while the nutritional regulation of the protein synthesis can occur quickly (i.e. in the postprandial period), most mechanisms involved in protein degradation will be adjusted rather slowly (hours or days) (Kimball and Jefferson 2002). Further controlled studies are needed to confirm the correlation between tissue protein turnover rates and RFI that is independent of the long-term effect of feeding level.

Finally, as previously justified, for the meal test, we only used the concentrate part of the two evaluated total mixed rations. These concentrates significantly differed in protein concentrations (118 vs 153 g metabolisable protein/kg DM) because they were designed to make the total mixed rations fed during the whole fattening period as iso-nitrogenous as possible (81.4 vs 85.6 g metabolisable protein/kg DM; Guarnido-Lopez et al., 2022). The disparity in protein content may have led to a more intense activation of the protein synthesis pathway in animals fed the high-starch concentrate. Nevertheless, since no significant interactions were identified between the concentrate composition and RFI for any of the analysed variables, similar conclusions could be anticipated even when the diets exhibit less pronounced contrasts in terms of nutritional values.

Conclusions

In contrast to our initial hypothesis, the findings from our study reveal that efficient RFI cattle demonstrated reduced muscle protein degradation and a tendency towards lower hepatic protein degradation and transaminase activity in response to the nutritional stimulus triggered by an identical meal size. These findings were irrespective of the composition of the two tested concentrates. Under the condition of this experiment, a biological association between protein degradation and amino acid catabolism was identified and could explain the greater nitrogen use efficiency reported in efficient RFI cattle. Regarding protein synthesis, our results indicate that regardless of the composition of the concentrate, when subjected to an identical single meal size prior to the evaluation efficient RFI cattle displayed a faster nutrient absorption in comparison to inefficient one. This enhanced response appeared to lead to a faster activation of muscle protein synthesis in efficient RFI cattle. Lower protein degradation and faster protein synthesis rates may account for the leaner carcasses typically observed in efficient RFI cattle.

Ethics approval

The protocol for this study was approved by the Ethics Committee of the Auvergne-Rhône-Alpes region and the French Ministry of Higher Education, Research and Innovation (Authorisation number: APAFIS #16194-2016101016361277 v6 delivered on 14th January 2019). The experiment was conducted at INRAE, Centre Auvergne-Rhône-Alpes, France.

Data and model availability statement

The data were not deposited in an official repository. Data are confidential but available to reviewers upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the corresponding author used Chat GPT in the writing process in order to improve English grammar. After using this tool/service, the author reviewed and edited the content as needed, and takes full responsibility for the content of the publication.

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Declaration of interest

None.

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