

Differential effects of milk proteins on amino acid digestibility, post-prandial nitrogen utilization and intestinal peptide profiles in rats

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- Differential effects of milk proteins on amino acid digestibility, post-prandial nitrogen
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- Nathalie Atallah¹²³, Claire Gaudichon¹, Audrey Boulier², Alain Baniel², Benoit Cudennec³, Barbara 4 Deracinois³, Rozenn Ravallec³, Christophe Flahaut³, Dalila Azzout-Marniche¹, Nadezda 5 Khodorova¹, Martin Chapelais¹, Juliane Calvez^{1*} 6 7 ¹UMR PNCA, AgroParisTech, INRAE, Université Paris-Saclay, 91123 Palaiseau, France ²Ingredia S.A. 62033 Arras Cedex, France 8 9 ³UMRt BioEcoAgro-INRAe 1158, Univ. Lille, Univ. Artois, Institut Charles Viollette, 59000 Lille, 10 France 11 * Corresponding author 12 13 Juliane Calvez. AgroParisTech-INRAE, UMR 914 PNCA, 22 place de l'agronomie, 91123 Palaiseau, France. Email: juliane.calvez@agroparistech.fr 14 15 Declarations of interest: B.C., B.D., C.F., C.G., D.A.M., N.K., M.C. and R.R. declare that they have 16 no conflict of interest. A.Ba., A.Bo. and N.A. are employed by Ingredia. 17 18 19 Abbreviations

AA: amino acid, AP: atom percent, APE: atom percent excess, EA-IRMS: elementary analyzer coupled to isotope ratio mass spectrometry, GC-MS: gas chromatography mass spectrometry, GC-C-IRMS: gas chromatography combustion isotope ratio mass spectrometry, FSR: fractional synthesis rate, MS: mass spectrometry, MS/MS: tandem mass spectrometry, N: nitrogen, n.s.: non-significant, QC: quality control, sem: standard error of the mean, UHPLC: ultra-high performance liquid chromatography.

26 Abstract

Objective: The aim of this study was to analyze the protein digestibility and postprandial
metabolism in rats of milk protein matrices obtained by different industrial processes.

Material and methods: The study was conducted on Wistar rats that consumed a meal containing different ¹⁵N-labeled milk proteins. Four milk matrices were tested: native micellar caseins (C1), caseins low in calcium (C2 low Ca²⁺), a matrix containing a ratio 63:37 of caseins and whey proteins (CW2) and whey proteins alone (W). Blood and urine were collected during the postprandial period and rats were euthanized 6 h after meal intake to collect digestive contents and organs.

Results: Orocaecal digestibility values of amino acids ranged between 96.0 \pm 0.2% and 96.6 \pm 0.4% for C1-, C2 low Ca²⁺- and W-matrices, while this value was significantly lower for CW2 matrix (92.4 \pm 0.5%). More dietary nitrogen was sequestered in the splanchnic area (intestinal mucosa and liver) as well as in plasma proteins after ingestion of W matrix, especially compared to the C1and C2 low Ca²⁺-matrices. Peptidomic analysis showed that more milk protein-derived peptides were identified in the caecum of rats after the ingestion of the matrices containing caseins compared to W matrix.

41 **Conclusion:** We found that demineralization of micellar caseins did not modify its digestibility and 42 postprandial metabolism. The low digestibility of the modified casein-to-whey ratio matrix may 43 be ascribed to a lower accessibility of the protein to digestive enzymes due to changes in the 44 protein structure, while the higher nitrogen splanchnic retention after ingestion of whey was 45 probably due to the fast assimilation of its protein content. Finally, our results showed that 46 industrial processes that modify the structure and/or composition of milk proteins influence 47 protein digestion and utilization.

48

49 **Keywords:** Milk proteins, digestibility, amino acid, rat, protein matrix

50 1. Introduction

In the past five decades, milk protein extraction has been widely applied at the dairy industry 51 level in order to use these proteins as food ingredients (Fox, 2001). Milk proteins are categorized 52 into two classes: caseins which represent 80% of the protein content in cow milk and whey 53 54 proteins which make up the remaining percentage (Wong, Camirand, & Pavlath, 1996). In their native state, both categories of proteins differ in their structure and physicochemical properties. 55 Whey proteins have a well-established globular structure and include β -lactoglobulin, α -56 lactalbumin and other minor fractions. Caseins have a flexible structure and exist in four types 57 that are more or less phosphorylated and glycosylated: α_{S1} -, α_{S2} -, β - and κ -caseins. The 58 particularity of caseins is their ability to interact together and with calcium phosphate to form 59 60 macromolecular aggregates named casein micelles. In contrast to whey proteins, the precise 61 structure of the casein micelle is still a subject of debate and numerous theories exist (Fox & 62 Brodkorb, 2008). However, it is generally accepted that the α_{s1} -, α_{s2} -, and β -caseins are found at the heart of the micelle and that κ-caseins, which are the least phosphorylated, are present at the 63 surface of the micelle, providing it stability and limiting its growth. It is also well-established that 64 calcium phosphate is necessary to maintain the integrity of the micelle (Dalgleish & Corredig, 65 2012a). 66

Some impacts of structural and physicochemical properties on the way that whey proteins and 67 68 caseins are digested have already been well demonstrated. Indeed, the notion of "slow" and 69 "fast" proteins was conceptualized when it was shown that caseins were digested in up to 6 h and 70 whey proteins in 2 h (Boirie et al., 1997; Mahe et al., 1996). This was explained by the ability of 71 caseins to form curds by acidic precipitation in the stomach, which delayed gastric emptying, 72 whereas whey proteins remained soluble in acidic conditions and were emptied rapidly from the stomach (Mahe et al., 1995). This fast digestion kinetics enhanced posprandial protein synthesis 73 74 (Boirie et al., 1997; Burd et al., 2012; Dangin et al., 2001; Tang, Moore, Kujbida, Tarnopolsky, & 75 Phillips, 2009) but also affected protein postprandial utilization (Lacroix, Bos, et al., 2006).

Evaluation of the protein quality implies its ability to satisfy the nitrogen and amino acid metabolic
 demands. It is now determined by its composition in indispensable amino acids and their

78 individual digestibility (FAO, 2013). The high nutritional quality of milk proteins is now well 79 established as they are rich in indispensable amino acids and have high digestibility values (Calvez et al., 2021; Deglaire, Moughan, Airinei, Benamouzig, & Tome, 2020; Gaudichon et al., 2002). 80 However, less is known about how the industrial processes affect digestibility and postprandial 81 utilization of milk proteins. A rat study, dedicated to impacts of milk heat treatments 82 demonstrated that the protein quality of microfiltered milk was maintained after different types 83 of heating, except for spray drying which decreased its digestibility (Lacroix, Leonil, et al., 2006). 84 85 Moreover, the type of applied processing modifies the protein structure,, notably for casein micelles that undergo demineralization. This demineralization process is achieved through pH 86 changes (acidification or alkalization) (Silva et al., 2013) or phosphorus and calcium chelation (de 87 Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2011). The acidification process, followed 88 by neutralization of the curd, results in sodium caseinates where the casein micelles have an 89 irregular shape and porous structure (Coskun, Sağlam, Venema, van der Linden, & Scholten, 2015) 90 compared to those obtained by membrane filtration that preserves their integrity (Dalgleish & 91 92 Corredig, 2012b). An in vitro study using a dynamic digestion model has shown that native micellar caseins and sodium caseinates were digested differently (Wang, Ye, Lin, Han, & Singh, 93 2018). Indeed, gastric hydrolysis of resolubilized milk powder containing the native form of 94 caseins was much slower than for sodium caseinates and that was related to its better coagulation 95 ability. Moreover, a static in vitro study has shown that demineralized and reticulated sodium 96 97 caseinates generated a different peptide pattern compared to native micellar caseins, suggesting an effect of protein structure on its digestion (Atallah et al., 2020). 98

Evidently, the industrial processing methods applied to generate milk protein matrices influence 99 their structure and composition, and thus the way that they are digested. Therefore, the aim of 100 our study was to evaluate the digestibility and postprandial utilization of ¹⁵N-labeled caseins and 101 whey proteins obtained by different industrial processes in rats. Milk protein matrices were 102 selected based on their different composition and/or their peptide heterogeneity during in vitro 103 104 digestion (Atallah et al., 2020). Four milk matrices were thus tested: native micellar caseins, demineralized micellar caseins (low in calcium), a matrix containing a modified ratio of micellar 105 106 casein to whey proteins (63:37) and whey proteins.

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107

108 **2. Material and methods**

109 **2.1.** Labeling and production of the milk protein matrices

110 The milk was labeled by Ingredia (Arras, France) by providing two cows with water containing 50 g of ¹⁵N ammonium sulfate [99%, (¹⁵NH₄)₂SO₄] twice a day for a period of 9 days. Collection of the 111 112 milk occurred from the fourth to the ninth day. An isotopic enrichment of 1.4 atom percent in the milk was obtained. The ¹⁵N-labeled milk was then pasteurized (75°C, 15 s) prior to protein 113 114 extraction, and a total of four protein matrices (Table 1) were obtained by filtration then 115 manufactured with different industrial processes by Ingredia (Arras, France). Two matrices were concentrated in native micellar caseins (C1 and C2 low Ca^{2+}) with C2 low Ca^{2+} having the lowest 116 calcium concentration. The CW2 matrix contained a lower casein-to-whey protein ratio (67:33) 117 compared to the one occurring naturally in cow milk (80:20) and the W matrix contained only 118 119 whey proteins. The amino acid composition of the four matrices is presented in supplemental 120 Table S1.

121

122 2.2. Animals and experimental design

123 The experimental protocol was implemented in compliance with the European Union directive 124 2010/63/EU for animal experiments and received an approval from the ethics committee in 125 animal experiments of INRAE Jouy-en-Josas (Comethea, registration number: 18-14) and the 126 French Ministry of Higher Education and Research (APAFIS n°15907-2018061823133762 v1). 127 Thirty-two male rats aged 5 weeks at their arrival were acquired from Envigo (Horst, The 128 Netherlands). After a week of adaptation to their new housing (Figure 1A), they were placed in 129 individual cages that contained wire-bottoms in order to avoid coprophagia. The rats were on a 130 usual 12 h/12 h dark-light cycle before being placed on a reversed dark-light cycle (dark period 131 07:00 to 19:00) at the beginning of the second week of the protocol. The rats were fed ad libitum 132 with a commercial laboratory chow during the first week and a standard diet (containing total 133 milk protein as the protein source; Table 2) during the second week. On the third week, the rats were subjected to an adaptation protocol for 1 week and randomly split into 4 groups (n = 134

135 8/group) according to the milk protein matrices they would consume: native micellar caseins (C1), native micellar casein low in calcium (C2 low Ca²⁺), casein and whey proteins in a 67:33 ratio 136 (CW2) and whey (W). The objective of this adaptation protocol (Figure 1B) was to get the rats 137 138 used to consuming a single meal in a limited time-frame as described previously (Atallah et al., 2022; Guillin et al., 2021; Tessier et al., 2020). This latter involved giving them access to a first 139 140 meal (weighing 4 g in dry weight) that had the same composition as the standard diet but that 141 contained the respective protein matrices in isonitrogenous amounts. They had access to this first 142 meal for 30 min (from 09:00 to 09:30) then to the standard diet ad libitum for 7 h (from 10:30 to 143 17:30). The rats were weighed every weekday, temperature was controlled and they had unlimited access to water during the whole experimental protocol. 144

On the day of euthanasia, the rats consumed the calibrated meals containing their respective ¹⁵N-145 labeled milk protein matrices. Blood was collected from the tail vein in the fasting state as well as 146 1 h and 3 h after meal ingestion, and plasma was then stored at -20°C. Thirty minutes before 147 euthanasia, the rats received an injection at the lateral vein of the tail of $[1-^{13}C]$ valine (Eurisotop, 148 Saint Aubin, France) at 150 µmol/100 g of body weight under gaseous anesthesia. Rats were 149 150 euthanazied 6 h after meal ingestion, after cardiac puncture (to collect plasma) under gaseous 151 anesthesia. Collection of the luminal contents in the stomach, small intestine, ileum (defined as the last 10 cm of small intestine before the caecum), caecum and colon was carried-out by rinsing 152 153 with NaCl (9%). Feces were gathered over the 6 h postprandial period. The contents were weighed 154 and stored at -20°C until freeze-drying. The mucosa of the duodenum, jejunum and ileum were scraped, frozen in liquid nitrogen and stored at -80°C. The gastrocnemius muscle and liver 155 samples were also collected, frozen in liquid nitrogen and stored at -80°C. Absorbent papers were 156 placed under the cages in order to retrieve urine during the 6 h postprandial period and the 157 158 bladder was punctured after euthanasia. The absorbent papers were then rinsed with distilled water and the urine eluates and from the bladder were pooled and stored at -20°C. 159

160

161 **2.3. Measurement of nitrogen and amino acid digestibility**

Protein- and amino acid-true digestibility was assessed following the ¹⁵N recovery and the ¹⁵Namino acid recovery in the digestive contents, respectively (Tessier et al., 2020). Briefly,

determination of the nitrogen percentage and ¹⁵N enrichment in the digestive contents and meals 164 was performed with an elemental analyzer (Vario Micro Cube, Elementar, Langenselbold, 165 Germany) coupled with isotopic ratio mass spectrometry (IsoPrime, GV Instruments Ltd., 166 Manchester, UK; EA-IRMS). Determination of the ¹⁵N-enrichment in each amino acid of digesta 167 and meals was obtained with gas chromatography (GC 6890 N, Agilent Technologies, Les Ulis, 168 169 France) coupled to an IsoPrime isotope ratio mass spectrometer (Isoprime, GV Instruments) via 170 the GC5 Isoprime interface (GC-C-IRMS). Analysis of amino acids in digesta and meals was 171 performed by ultra-high-performance liquid chromatography (UHPLC) using the AccQTag Ultra method (Waters, France), as previously described (Guillin et al., 2021). 172

Dietary nitrogen quantity (N_{diet}, mmol) recovered in the different digestive content was
 determined using the formula as follows:

175
$$N_{diet \, digesta} = N_{tot} \times \frac{APE_{digesta}}{APE_{meal}}$$

Where N_{tot} represents the total amount of nitrogen in the sample (mmol) and APE the enrichment
 excess of ¹⁵N in the sample and meal. APE is defined as the enrichment of the sample in atom
 percent (AP) minus the natural enrichment.

Orocaecal digestibility (%) was used as a proxy of ileal digestibility (Atallah et al., 2022; Guillin et al., 2021; Tessier et al., 2020) in order to have enough digesta for the determination of isotopic enrichment. Digesta collection was performed 6 h after ingestion, a compromise between complete digestion and minimal duration of fermentation of digesta in the caecum. True orocaecal nitrogen digestibility (%) was calculated as follows:

184 True nitrogen orocaecal digestibility =
$$100 \times \frac{N_{ing} - (N_{diet\,ileum} + N_{diet\,caecum})}{N_{ing}}$$

Where N_{ing} is the amount of nitrogen ingested by the rats (mmol). True orofecal nitrogen digestibility was also determined considering nitrogen losses in the colon and feces, in addition to losses in the ileum and caecum. Similarly to true orocaecal nitrogen digestibility, true orocaecal digestibility for each individual amino acid was determined by estimating the dietary amino acids not absorbed in the intestinal tract and recovered in the digesta. 190

191 **2.4. Measurement of nitrogen postprandial utilization**

192 Total urea in urine and plasma was estimated by the urease-Berthelot's method (Urea assay, Randox, Crumlin, UK). Plasma was separated in protein-, free amino acid- and urea-fractions as 193 194 described previously (Bos et al., 2005). Urea was assumed to be uniformly distributed throughout 195 the total body water and dietary nitrogen amount recovered in the urea body pool (mmol) as 196 described previously (Lacroix, Leonil, et al., 2006). We assumed that the urinary ¹⁵N-enrichment was a proxy of the urinary urea ¹⁵N-enrichment and calculated the dietary nitrogen amount 197 198 recovered in the urinary as described previously (Lacroix, Leonil, et al., 2006). The total amount 199 of dietary nitrogen transferred to urea was obtained from the sum of the dietary nitrogen amount 200 excreted in urinary urea and the one recovered in the body urea pool. The dietary nitrogen amount recovered in proteins of organs or plasma (mmol) was calculated as described previously 201 202 (Tessier et al., 2019). The dietary nitrogen amount recovered in the different tissues was 203 calculated with respect to organ weight while the plasma was assumed to represent 3.5% of body weight for plasma protein calculation. (Waynforth & Flecknell, 1992). 204

The ¹⁵N-enrichment in plasma fraction, urine, organs and intestinal mucosae was measured by EA-IRMS as described previously (Lacroix, Leonil, et al., 2006). For the different mucosae tissues, values were expressed as %/100 mg of tissue.

208

209 **2.5. Measurement of** *in vivo* protein synthesis in muscle and liver

In vivo fractional synthesis rates (FSR) of proteins in the gastrocnemius muscle and liver were
 determined using the [¹³C]-valine flooding dose method as previously described (Chevalier et al.,
 2013; Oberli et al., 2015). Briefly, the protein-bound and free amino acid fractions were separated
 in each tissue. [¹³C]-valine enrichment in the free amino acid fraction was estimated by gas
 chromatography (GC 6890N, Agilent Technologies) coupled to mass spectrometry (MS 5973N,
 Agilent Technologies) (GC-MS). Enrichment in [¹³C]-valine in protein bound amino acid fraction

was analyzed using a GC-C-IRMS. The FSR of proteins (expressed in %/day) in the liver and muscle
was calculated as described previously (Tessier et al., 2019).

218

219 2.5. Measurement of plasma amino acid kinetics

Plasma concentration of free amino acids, 0, 1, 3 and 6 h after meal ingestion, was determined,
after protein precipitation, by UHPLC with AccQTag Ultra method (Waters) as previously
described (Calvez et al., 2021).

223

224 **2.6.** Analysis of peptidomic profil in cecal digesta

225 For peptidomics analysis, digestive samples obtained from rats (n = 3) fed a protein-free meal in 226 a study with comparable design (Guillin et al., 2021) was included as control. Peptide sequence 227 identifications in the caecal digesta samples were performed as previously described with some 228 minor changes (Atallah et al., 2020). The dried caecum samples were rehydrated at a concentration of 10 mg.mL⁻¹ in water containing 0.1% trifluoroacetic acid (TFA) and centrifuged. 229 Peptides were concentrated ten times and desalted with C18 solid phase extraction (SPE) columns 230 (Bond Elut C18 1000 mg minicolumns, Agilent Technologies). Dried peptides were dissolved in 231 232 ultra-pure H₂O containing 0.1% TFA (v/v), centrifuged and supernatants were analyzed in 233 triplicate by reverse phase-high performance liquid chromatography coupled to tandem mass 234 spectrometry (RP-HPLC-MS/MS). Peptides were chromatographically separated at 30°C on an 235 Acquity UPLC system (Waters Corporation, France) using a C18AQ column (150 × 3.0 mm, 2.6 μm, 236 Uptisphere CS EVOLUTION, Interchim, France). The mobile phases consisted of solvent A (0.1% 237 formic acid / 99.9% water, (v/v)) and solvent B (0.1% formic acid / 99.9% (v/v) acetonitrile (ACN), (v/v)). The ACN gradient (flow rate 0.5 mL.min⁻¹) was as follows: from 1% to 40% solvent B over 238 239 22.5 min, from 40% to 95% solvent B over 2.5 min followed by washing and equilibrating procedures with 95% and 1% solvent B for 2.5 min each, respectively. The eluate was injected 240 into the electrospray ionization source of a qTOF Synapt G2-Si[™] (Waters). Database searches 241 242 were performed using the UniProt database restricted to Bos taurus organism via PEAKS® Studio XPro software (Bioinformatics Solutions Inc., Waterloo, Canada). The peptide identity searches
were performed without notifying the choice of enzyme.

245

246 2.7. Statistical analyses

A power calculation was performed to determine the sample size required to detect significant differences with a statistical power of 95% and α level set at 0.05. According to former studies, interindividual variability in labelled protein digestibility measured at caecal level in rats was around 0.8% (Lacroix, Leonil, et al., 2006; Oberli et al., 2016). We considered that a difference in digestibility of different milk protein matrices > 1.5% would be physiologically pertinent. The sample size was thus calculated to be 8 rats per group (G*Power 3.1).

253 All results are expressed as mean ± sem. Statistical analyses were performed on GraphPad Prism 254 8.2.1 and R (version 3.5.2). Normality of data was tested with Quantile vs Quantile Plots and Shapiro-255 Wilk tests. The effect of the group was tested using a one-way ANOVA and post hoc Tukey tests were applied for pairwise comparisons. Mixed linear models with repeated measures were 256 performed to test differences between groups over time. Differences were considered 257 statistically significant with a p-value < 0.05. For peptidomic analysis, a multiple factor analysis 258 (MFA) was conducted to comparing data from MS analysis (α S1-casein and β -casein heat maps). 259 260 MFA result was represented as map of individuals (similarity and discrepancy between 261 individuals). MFA was performed using the R software (version 4.2) and the FactorMineR 2.6 package (Lê, Josse, & Husson, 2008). 262

263

264 **3. Results**

265 **3.1. Body weight and food intake**

No difference in body weight gain and food intake (data not shown) was observed between the 4 groups of rats. The final body weights were 291.8 \pm 5.8 g, 291.8 \pm 5.0 g, 288.0 \pm 5.8 g and 290.3 \pm 6.1 g for the rats consuming the C1-, C2 low Ca²⁺-, CW2- and W-milk protein matrix, respectively.

269

270 **3.2. Dietary nitrogen recovery**

Figure 2 represents the quantity of dietary nitrogen collected in the different portions of the gastrointestinal tract according to the milk protein matrix ingested by the rats. Whatever the matrix, the majority of the dietary nitrogen was recovered in the caecum of rats (Figure 2). Therefore, in the caecum, a significant effect of the matrix was observed (P = 0.0046) with a significantly higher amount of dietary nitrogen after ingestion of CW2-matrix in comparison to C1 (P = 0.0377), C2 low Ca²⁺ (P = 0.0032) and W (P = 0.0363). No significant difference between the milk protein matrices was noted in the other segments of the gastrointestinal tract.

278

279 3.3. Digestibility of nitrogen and amino acids

280 Nitrogen- and individual amino acid-digestibility values of the milk protein matrices are presented 281 in **Table 3**. High values of nitrogen digestibility were found for the 4 milk protein matrices ranging 282 from 93.5 \pm 0.3% (for CW2) to 95.5 \pm 0.3% (for C2 low Ca²⁺) for orocaecal digestibility. Orocaecal nitrogen digestibility was significantly lower for the CW2 matrix in comparison to C1 (P = 0.0405), 283 C2 low Ca²⁺ (P = 0.0016) and W (P = 0.0094). The same tendency was observed with the orofecal 284 285 nitrogen digestibility. Regarding individual amino acid digestibility values, their average orocaecal digestibility varied from 92.4 ± 0.5% (for CW2) to 96.6 ± 0.4% (for C1), and the same tendency 286 287 was observed for the CW2 matrix, which had a significantly lower value compared to the other 288 matrices (P < 0.0001). Indeed, the orocaecal digestibility was lower for the CW2 matrix for most 289 of the individual amino acids except for methionine, alanine, glycine, and serine.

290

3.4. Peptide identification in the caecum

Figure 3 gathers the different data obtained by peptidomics analysis of the caecal digesta 6h after ingestion of C1-, C2 low Ca²⁺-, CW2- or W-matrices (n = 8 rats/group).

As illustrated in Figure 3A (upper panels), whatever the milk protein matrix used, the average number of MS- and MS/MS-scans recorded during the HPLC-MS/MS runs were 2,091 ± 340 MS scans and 6,452 ± 675 MS/MS scans, respectively. The One-way ANOVA multiple comparisons (P 297 < 0.05) carried out from the mass spectrometry data revealed no statistically significant differences between groups. In contrast, the number of peptides identified by MS/MS-data 298 confrontation to Bos taurus protein database using PEAKS® Studio XPro (Figure 3A (bottom 299 300 panels)) were statistically different as highlighted by the lowercase letters. Indeed, the number of identified peptides was significantly more important from caecum samples corresponding to 301 302 C1-, C2 low Ca²⁺- and CW2- compared to the W-matrix with, for 8 biological replicates, 51 ± 36 , 303 38 ± 18 , 35 ± 21 and 6 ± 6 , respectively. Among these identified peptides, a large majority 304 corresponds to milk protein peptides and only casein peptides: 36 ± 14 , 36 ± 18 , 32 ± 20 and 5 ± 100 6, respectively. The full list of all identified casein peptides for each experimental condition is 305 306 presented in supplementary Table S2. Interestingly, no peptide from kappa-casein nor whey proteins were identified from the 8 biological replicates whatever the milk protein matrix used. 307 Moreover, as expected, the few peptides identified from the caecum samples of control rats were 308 309 not milk but cellular protein-derived peptides (data not shown). Moreover, it is necessary to 310 precise that no milk protein-derived peptides were identified in the duodenum nor in the jejunum 311 which attests that the digestion was finished 6 h after meal intake.

Heat maps (Fig. 3B), displaying the amino acid occurrences in α S1- and β -casein according to the ingestion of C1-, C2 low Ca²⁺-, CW2- or W-matrices, show the protein-backbone zones which were resistant to gastrointestinal digestion and intestinal absorption. For α S1-casein, except for the Wmatrix where no casein peptides were identified, the same resistant zones were found after ingestion of C1-, C2 low Ca²⁺- and CW2-matrices. The resistant zones were distributed over the N-terminal half of the protein skeleton. For β -casein, overall, only one main N-terminal zone resistant to gastrointestinal digestion emerges for all ingested matrices.

The 2D-plot graph corresponding to the first two dimensions issued from the MFA, constructed from all identified α S1- and β -casein peptides of each individual (Fig. 3C), highlights the similarity and discrepancy between the samples. The green plots related to the ingestion of whey proteins were distinctly different from the others, except for one (over the 8) of each of the 3 casein matrices. Moreover, whatever the ingested-casein matrix, no set of individuals was different from the two others. 325

326 **3.5. Postprandial distribution of dietary nitrogen**

The incorporation of dietary nitrogen into the body and urinary urea pools, organs and digestive 327 tissues 6 h after meal ingestion, according to the different rat groups, is presented in **Table 4**. No 328 significant difference was observed between the matrices regarding the quantity of dietary 329 nitrogen transfer to body or urinary urea pool and consequently, no difference was found on the 330 total deamination with values ranging from 7.8 ± 2.1% (for the C2 low Ca²⁺) to 11.3 ± 4.8% (for the 331 CW2 group). A significant effect of group was found on the incorporation of dietary nitrogen into 332 333 the liver (P < 0.0001) and gastrocnemius muscle (P = 0.0006) 6 h after meal. The group of rats consuming the W matrix showed a significantly higher incorporation of dietary nitrogen in both 334 liver (P < 0.0001 for C1 and C2 low Ca²⁺, and P = 0.0066 for CW2) and gastrocnemius muscle (P = 335 0.0232 for C1, P = 0.0003 for C2 low Ca^{2+} and P = 0.0462 for CW2) in comparison to the other 336 groups. The incorporation of dietary nitrogen into the digestive mucosae was not different in the 337 duodenum, but differences between groups were found in the more distal parts of the small 338 339 intestine. Indeed, significantly higher dietary nitrogen was found after ingestion of CW2 in the jejunum in comparison to C1 (P = 0.0331) and C2 low Ca^{2+} (P = 0.0182) and even higher after 340 ingestion of the W matrix in comparison to the 3 others (P < 0.0001 for C1 and C2 low Ca²⁺, and P 341 = 0.0372 for CW2). In the ileum, incorporation of dietary nitrogen was significantly higher after 342 ingestion of matrices containing whey (CW2 and W) in comparison to the C1 (P = 0.0088 and < 343 0.0001, respectively) and C2 low Ca^{2+} (P = 0.0001 and < 0.0001, respectively). 344

345

346 3.6. Postprandial incorporation of dietary nitrogen to plasma protein

The incorporation of dietary nitrogen to plasma proteins was evaluated according to the different matrices over the 6 h-postprandial period (**Figure 4**). Significant effects of groups, time and their interaction (P < 0.0001) were observed during the whole postprandial period. Starting from 3 h after ingestion, the W matrix started to differentiate from the other matrices as more dietary nitrogen was incorporated to plasma proteins compared to CW2 (P = 0.0094), and both C1 and C2 low Ca²⁺ (P < 0.0001). Interestingly, 6 h after ingestion, the W matrix still showed the highest incorporation of dietary nitrogen compared to other matrices (aP < 0.0001 for both, CW2, C1 and C2 low Ca²⁺. The CW2 matrix also incorporated significantly more dietary nitrogen into plasma protein compared to the C2 low Ca²⁺ 3 h and 6 h after meal ingestion (P = 0.0009 and P = 0.0029, respectively). During the entire postprandial period, no difference between the casein-based matrices, C1 and C2 low Ca²⁺, were observed.

358

359 3.7. In vivo protein synthesis

Figure 5 shows the FSR of proteins, after the 6-h postprandial period, in the liver and gastrocnemius muscle according to the different milk protein matrices ingested. No significant difference between the protein matrix groups was pointed out in either the liver or the muscle.

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364 **3.8. Postprandial plasma amino acid kinetics**

365 We determined the kinetics of plasma amino acid concentrations from the fasting state to 6 h 366 after ingestion of protein matrices (Figure 6). A significant effect of time was observed for the 367 kinetics of total- (P = 0.0197) and indispensable-amino acid (P < 0.0001) concentrations, as well as leucine (P < 0.0001) concentration kinetics, with an increase in amino acid concentrations after 368 369 meal ingestion and a return to basal level at 6 h. The casein-based matrices (C1 and C2 low Ca²⁺) 370 exhibited a prolonged plateau over 6 h after meal ingestion, however no significant effect of groups was observed regarding kinetics of total- or indispensable-amino acid concentrations. In 371 372 contrast, plasma leucine concentration kinetics was influenced by groups (group x time effect: P < 0.0001) and ingestion of the W matrix induced a significantly higher increase of leucine 373 concentration 1 h after meal intake in comparison to C1 (P = 0.0021) and C2 low Ca²⁺ (P = 0.0034). 374

375

376 4. Discussion

The aim of this study was to characterize the digestibility and postprandial utilization of ¹⁵Nlabeled milk protein matrices, obtained by different industrial processes, *in vivo*. The matrices included two micellar casein-based matrices with varying calcium concentrations (C1 and C2 low
 Ca²⁺), a modified ratio of whey-to-micellar casein matrix (CW2) and a whey-based matrix (W).

381 Milk proteins are considered as high-quality proteins and are therefore known to display amongst 382 the highest digestibility values for dietary proteins. Accordingly, total milk protein, casein or whey protein isolate exhibited ileal nitrogen digestibility from 91 to 95% and mean amino acid ileal 383 384 digestibility values from 92 to 97% in humans (Calvez et al., 2021; Gaudichon et al., 2002; Guillin 385 et al., 2022). In rats, ileal nitrogen or amino acid digestibility values varied between 92 and 96%, depending on the protein matrix, with higher values for whey (98 - 100%) (Lacroix, Leonil, et al., 386 387 2006; Rutherfurd, Fanning, Miller, & Moughan, 2015). Our results are partly in accordance with 388 these previously published studies as the nitrogen orocaecal digestibility (a proxy of ileal digestibility) of the casein-based matrices were 94.9% and 95.5% for native caseins (C1) and 389 390 caseins with low calcium (C2 low Ca²⁺), respectively. We found comparable results for the native 391 casein matrix in a previous study (Atallah et al., 2022). However, we observed a slightly lower 392 digestibility of whey protein (95.2% and 96.6% for nitrogen and mean amino acid orocaecal digestibility, respectively) in comparison to previously published data (Lacroix, Leonil, et al., 2006; 393 394 Rutherfurd et al., 2015). These slight discrepancies may be due to differences in methodology or 395 in the whey protein used. We also studied a milk protein matrix that had a modified whey-to-396 micellar casein ratio (63: 37; CW2). Surprisingly, the rats consuming this matrix displayed a higher 397 amount of dietary nitrogen in the caecum 6 h after ingestion, resulting in a slightly lower 398 orocaecal digestibility for both nitrogen (93.5%) and mean amino acid (92.4%) in comparison to 399 all the other matrices. The digestibility observed for this matrix was lower than that reported for 400 total milk proteins in the literature. Indeed, total milk proteins which contain a casein-to-whey 401 ratio of 80:20 had a true orocaecal nitrogen digestibility of 96% in rats (Lacroix, Leonil, et al., 402 2006). An increase in the whey protein content could have possibly led to additional interactions between whey proteins and caseins which resulted in structural changes that limited the 403 accessibility to digestive enzymes in the CW2 matrix. Such an hypothesis is supported by the 404 405 higher amount of free thiol groups available from β -lactoglobulin which can lead to an increase in the formation of disulfide bonds between caseins and whey proteins (Zhao et al., 2016). 406

407 The duodenal, jejunal, ileal and caecal digesta were submitted to peptidomics analysis combining 408 an RP-HPLC-MS/MS analysis and the bioinformatic identification of peptides. Only the ileum and caecum contents contained milk protein-derived peptides which belong to the same parent 409 410 proteins. Since the number of identified peptides was higher in the caecal digesta, we decided to deeply analyse the results obtained for them. The non-statistically different number of MS- and 411 412 MS/MS-scans recorded during the RP-HPLC MS/MS runs indicates that the number of ions, their 413 intensity or both chosen for fragmentation in these samples are no more important in one run 414 compared to others runs, and suggests, as previously reported that the mass spectrometry data are fully comparable (Atallah et al., 2020). First of all, the overall number of peptides identified 415 from caecal content ranges from 50 ± 35 to 6 ± 5 according to the milk matrix ingested, and the 416 number of identified peptides was significantly lower in the caecal digesta for the whey matrix 417 compared to matrices containing mainly caseins. Recently, Sanchón et al. reported, the 418 419 identification of 415 and 230 different peptides from human jejunal contents after casein and 420 whey intake, respectively (Sanchon et al., 2018). Although human and rat models may not be 421 directly comparable, the lower number of identified peptides from the rat caecum content 422 correlates with the absorptive intestinal function of the small intestine. Moreover, the whey 423 proteins, especially β -lactoglobulin, are reported to be resistant to pepsin action (Bouzerzour et 424 al., 2012) but are completely hydrolyzed after 60 min of intestinal digestion (Sanchon et al., 2018). In our study, no peptides from the whey proteins were identified in the caecum whatever the 425 426 matrix ingested, suggesting the full gastrointestinal digestion and the full intestinal absorption of 427 whey peptides. Inversely, although caseins are hydrolyzed within the first minutes of pepsin 428 hydrolysis (Dupont & Tomé, 2020), some casein peptides are resistant to gastrointestinal 429 digestion (Egger et al., 2017) and to epithelial proteases (Bauchart et al., 2007), and consistently, 430 were found in the caecum content of 29 rats in our study. The gastrointestinal digestion resistance 431 of such peptides is generally attributed to proline-containing peptides. In our study, among all the 432 digestion-resistant peptides identified in the caecum, the occurrence of glutamate, valine, 433 isoleucine, proline and serine were 330, 166, 118, 118 and 107, respectively. Numerous serine 434 residues are annotated as phosphorylated by the peptide identification software (Peaks[®] studio XPro). This latter point agrees with the results reported by Sanchón *et al.* (Sanchon et al., 2018). 435

436 Finally, the protein backbone of α s1-casein resistant to the gastrointestinal tract was more 437 extensive but stayed in the N-term half while the β -casein one was mainly located in the N-term 438 part.

439 An interesting characteristic amongst the different milk proteins is that the caseins are slowly digested in contrast to whey proteins that are rapidly digested. This has been demonstrated 440 441 previously with differences in plasma amino acid concentration profile (Boirie et al., 1997; Lacroix, 442 Bos, et al., 2006) with a higher increase in plasma amino acid concentration after whey isolate ingestion in comparison to casein in humans (Lacroix, Bos, et al., 2006). We did not clearly observe 443 444 this plasma amino acid concentration profile in our study after ingestion of the different milk 445 protein matrices. This may be due to the lipids and carbohydrate included in the meal that could 446 have minimized potential kinetics differences (Gaudichon et al., 1999) or to the animal model. However, the rats that consumed the whey protein (W matrix) meal exhibited a plasma leucine 447 concentration increase 1 h after ingestion in comparison to the two other casein-based matrices 448 (C1 en C2 low Ca²⁺), whereas no difference was observed with the CW2 matrix. The whey protein 449 matrix presents the highest leucine content so it triggers a more massive leucine appearance 450 451 compared to caseins, as shown in human and rodent plasma (Kanda et al., 2016; Tang et al., 2009). 452 Consistently, the moderately high leucine content of the CW2 matrix together with its lower digestibility resulted in a slight plateau-type increase of plasma leucine whereas no increase was 453 454 observed for the casein matrices.

Thanks to ¹⁵N-labelling of the milk protein matrices, we evaluated the incorporation of dietary 455 456 nitrogen in the splanchnic area (intestinal mucosa, liver), in the plasma proteins (mainly composed of exported liver proteins) and in periphery, in the muscle. At the end of the 6 h 457 458 postprandial period, the rats consuming the whey protein matrix presented a higher 459 incorporation of dietary nitrogen into the splanchnic area, plasma protein and in periphery. The 460 increased incorporation in the plasma proteins was also observed as soon as 3 h after whey protein intake. The higher splanchnic and peripheral uptakes of dietary nitrogen from whey were 461 probably linked to the higher speed of digestion of whey proteins. In contrast, in a previously 462 published study (Lacroix, Leonil, et al., 2006), no difference was found between whey protein and 463 micellar caseins in terms of dietary nitrogen transfer to the liver and medial or distal intestinal 464

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465 mucosa of rats 6 h after meal intake. These different results could be explained by the difference 466 in processing method used to obtain the milk protein powders, which could have in turn affected 467 the protein structure (van Lieshout, Lambers, Bragt, & Hettinga, 2020). We also determined the 468 transfer of dietary nitrogen to body and urinary urea. We reported a postprandial deamination of milk proteins ranging from 8 to 11% and no difference between the milk protein matrices. 469 470 Values of postprandial deamination ranging from 8% to 20% have been previously reported 471 depending of the size of the meal, the habitual protein intake and the time after meal ingestion 472 in rats (Lacroix, Leonil, et al., 2006; Morens et al., 2000). Had we evaluated the postprandial kinetics of dietary nitrogen transfer to urea, a difference might have been observed, since transfer 473 of dietary nitrogen to urea was quicker for whey proteins and reached a maximum 2 h after 474 ingestion compared to caseins or total milk proteins, as reported in a human clinical trial (Lacroix, 475 Bos, et al., 2006). 476

477 Despite higher incorporation of dietary nitrogen in muscle and liver, and a peak of plasma leucine concentration after whey proteins ingestion, no difference in terms of protein synthesis was 478 479 found in the gastrocnemius muscle and the liver compared to the other matrices. We found a FSR 480 of proteins of around 100 - 110%/day in the liver and around 5 - 7%/day in the muscle 6 h after 481 ingestion of the four milk protein matrices, as previously observed in rats (Chevalier et al., 2013; Kanda et al., 2016; Tessier et al., 2019). Similarly, no difference in muscle and liver FSR of proteins 482 483 was previously observed in energy-restricted rats that consumed meals containing either caseins 484 or whey proteins, or a 50/50 mixture of both proteins (Adechian, Remond, Gaudichon, Dardevet, & Mosoni, 2011; Adechian et al., 2012). In humans, the effects of different milk proteins on the 485 stimulation of muscle protein synthesis seemed to be highly dependent on the structure of the 486 matrix and the composition in amino acids of the milk proteins (Reitelseder et al., 2011; Tang et 487 488 al., 2009; Tipton et al., 2004). However, differences could have been pointed out if the FSR of proteins had been measured at different time points. Indeed, in a study evaluating protein 489 anabolism after different milk protein consumption in rats, the muscle protein FSR measured 4 h 490 491 after ingestion was similar after the whey protein, the caseinate and the milk protein intake, but time-course of FSR time varied among the different protein types (Kanda et al., 2016). 492

493

494 **5. Conclusions**

All the milk protein matrices tested in this study had high digestibility values. However, our results 495 496 showed that milk proteins with a modified whey-to-micellar casein ratio displayed a slightly lower 497 digestibility in comparison to caseins or whey proteins, probably due to an alteration in the 498 structure of the matrix induced by a rearrangement of proteins. No difference on digestibility and postprandial metabolism was observed after ingestion of micellar caseins and caseins with low 499 500 calcium content, showing that structural changes linked to demineralization of caseins did not 501 modify the nutritional quality of caseins. Despite similar digestibilities in both the casein-based and whey matrices, a higher incorporation of dietary nitrogen in most tissues was found, in 502 503 accordance with the rapid kinetics of whey proteins. Additionnally, no whey protein-derived 504 peptides were identified in the rat caecal digesta. Indeed, the identified peptides, resistant to 505 gastrointestinal digestion, belong only to α s1-, α s2- and β -caseins and are mainly located in the 506 N-terminal half of the α s1-casein and the proximal N-term region of the β -casein. Hence, our 507 results show that modification of the structure and composition of the milk proteins by industrial processes influence protein digestion and utilization. Knowledge of the consequences of 508 509 industrial processes on protein digestion and quality is thus critical to characterize the nutritional 510 properties of innovative proteins.

511

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515

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524

525 **Conflict of interest**

B.C., B.D., C.F., C.G., D.A.M., J.C., M.C., N.K. and R.R. declare that they have no conflict of interest.
N.A., A.Ba., and A.Bo. are employed by Ingredia.

528

529 Authorship

The authors' responsibilities were as follows: J.C., C.G., A.Ba. and A.Bo. contributed to the conception and design of the study; N.A., J.C. conducted the research; N.A., J.C., N.K., M.C., contributed to sample analyses, N.A. and J.C. analyzed the data; B.C, B.D. C.F. and R.R conducted the peptitomic analysis of the digestive contents; N.A. wrote the original draft of the paper; all authors reviewed and edited the manuscript; C.G. administered the project, and all authors read and approved the final manuscript.

536

537 Data statement

538 Data described in the manuscript will be made available upon reasonable request, pending 539 application and approval.

540 Tables

	Native micellar casein (%)	Whey protein (%)	Calcium (%)	Total protein content (%)
C1	92	8	2.6	83
C2 low Ca ²⁺	92	8	1.6	85
CW2	63	37	1.8	81
W	< 1	> 99	0.3	81

541 Table 1. Characteristics of the milk protein matrices

542 C: casein; W: whey. The matrices C1 and C2 low Ca²⁺ are casein-based but differ in their calcium concentration and
 543 the structure of casein micelles, while the CW2 matrix contains both casein and whey proteins in lower proportion
 544 as in cow milk. The matrix W is entirely composed of whey proteins. The total protein content was based on
 545 nitrogen content x 6.38.

546

547 Table 2. Composition of standard and experimental diets

	Standard and experimental diets (g/kg)
Protein*	140
Starch	622
Sucrose	100
Soya bean oil	40
Mineral mix	35
Vitamin mix	10
Cellulose	50
Choline	3
Protein (% Energy)	14
Carbohydrates (% Energy)	75
Lipids (% Energy)	11

548 * The protein source was the total milk proteins for the standard diet and correspond to the milk protein matrices

549 in the experimental diets.

550

551 Table 3. Amino acid and nitrogen digestibility of the different milk protein matrices

	C1	C2 low Ca ²⁺	CW2	W	P-value	
Indispensable AA orocaecal digestibility (%)						
Isoleucine	96.0 ± 0.5^{a}	95.5 ± 0.3ª	92.5 ± 0.5 ^b	96.0 ± 0.4^{a}	< 0.0001	
Leucine	97.4 ± 0.2^{ac}	97.4 ± 0.2^{a}	93.5 ± 0.5 ^b	96.1 ± 0.4 ^c	< 0.0001	
Lysine	97.7 ± 0.3ª	97.6 ± 0.3^{a}	94.5 ± 0.4^{b}	94.4 ± 0.7^{b}	< 0.0001	
Methionine	96.9 ± 0.3^{a}	97.5 ± 0.1^{a}	96.7 ± 0.3ª	98.1 ± 0.2^{b}	0.0033	
Phenylalanine	97.5 ± 0.6 ^a	97.6 ± 0.2^{a}	94.3 ± 0.7 ^b	97.5 ± 0.2ª	< 0.0001	

Threonine	97.4 ± 0.2^{a}	97.4 ± 0.1^{a}	96.5 ± 0.3 ^b	97.8 ± 0.2^{a}	< 0.0001		
Valine	96.6 ± 0.4^{a}	96.0 ± 0.2^{a}	90.6 ± 0.7 ^b	96.2 ± 0.4^{a}	< 0.0001		
Dispensable AA orocaecal digestibility (%)							
Alanine	98.6 ± 0.3 ^a	97.0 ±0.3 ^b	98.0 ± 0.3^{ab}	98.2 ± 0.3^{ab}	0.0064		
Aspartate + asparagine	97.3 ± 0.3ª	95.8 ± 0.3^{a}	90.0 ± 0.6^{b}	97.2 ± 0.3ª	< 0.0001		
Glycine	97.4 ± 0.4	97.4 ± 0.3	96.7 ± 0.3	97.5 ± 0.2	n.s.		
Glutamate + glutamine	96.0 ± 0.6^{a}	94.7 ± 0.5^{a}	87.9 ± 0.8 ^b	97.0 ± 0.5ª	< 0.0001		
Proline	94.8 ± 0.6 ^a	94.0 ± 0.6 ^{ab}	91.4 ± 1.2 ^b	95.7 ± 0.4^{a}	0.0037		
Serine	95.5 ± 0.7	95.5 ± 0.4	95.3 ± 0.4	96.7 ± 0.4	n.s.		
Tyrosine	98.8 ± 0.1^{a}	98.5 ± 0.1^{a}	96.5 ± 0.3 ^b	98.4 ± 0.2^{a}	< 0.0001		
Average AA digestibility ¹ (%)	96.6 ± 0.3 ^a	96.0 ± 0.2^{a}	92.4 ± 0.5 ^b	96.6 ± 0.4^{a}	< 0.0001		
Nitrogen digestibility (%)							
Orocaecal digestibility	94.9 ± 0.4 ª	95.5 ± 0.3 °	93.5 ± 0.3 ^b	95.2 ± 0.3 ª	0.0019		
Orofecal digestibility	93.9 ± 0.4^{a}	94.4 ± 0.3 ª	92.1 ± 0.4 ^b	94.2 ± 0.3 ª	0.0004		

Values are mean \pm sem, n = 8 rats/group. ¹Average digestibility was calculated from the mean amino acid digestibilities weighted by the proportion of each amino acid in the milk protein matrix. The effect of the protein matrix was tested with a one-way ANOVA model. Values with different letters within the same row are statistically different (P < 0.05). n.s, not significant. AA, amino acid.

556

557 Table 4. Transfer of dietary nitrogen to the urea pool and to the liver and gastrocnemius muscle

558 (expressed as % of ingested nitrogen) 6 h after ingestion of different protein matrices.

	C1	C2 low Ca ²⁺	CW2	W	P value
Body urea pool	2.5 ± 0.4	2.6 ± 0.5	3.2 ± 0.7	2.5 ± 0.5	n.s.
Urinary urea pool	5.9 ± 0.8	5.2 ± 0.7	8.1 ± 1.3	6.7 ± 0.5	n.s.
Total deamination	8.4 ± 0.9	7.8 ± 0.8	11.3 ± 1.7	9.2 ± 0.7	n.s.
Liver ¹	12.6 ± 0.7ª	11.4 ± 0.5^{a}	14.8 ± 1.4ª	19.5 ± 0.7 ^b	< 0.0001
Gastrocnemius muscle1	0.50 ± 0.03 ^a	0.41 ± 0.02^{a}	0.51 ± 0.04ª	0.65 ± 0.02^{b}	0.0006
Duodenum ²	0.29 ± 0.01	0.31 ± 0.03	0.33 ± 0.02	0.34 ± 0.02	n.s.
Jejunum ²	0.27 ± 0.02 ^a	0.26 ± 0.01^{a}	0.35 ± 0.02^{b}	$0.42 \pm 0.02^{\circ}$	< 0.0001
lleum ²	0.13 ± 0.01^{a}	0.11 ± 0.01^{a}	0.17 ± 0.01^{b}	0.19 ± 0.01^{b}	< 0.0001

559 Values are mean ± sem, n = 8 rats /group. ¹ expressed as % of ingested nitrogen /organ. ² expressed as % of dietary

560 nitrogen incorporated in 100 mg of mucosae. The effect of the protein matrix was tested with a one-way ANOVA

561 model. Values with different letters within the same row are statistically different (P < 0.05). *n.s*, not significant.

562 Legends of figures

Figure 1. Experimental protocol of the study. (A) Overall planning of the 3-week animal experiment. (B) Daily alimentary protocol to accustom the rats to consume a single calibrated meal.

566

Figure 2. Dietary nitrogen (expressed as % of ingested nitrogen) recovered in gastrointestinal tract contents according to the milk protein matrix. Values are mean \pm sem, n = 8 rats/group. The group effect was tested using a one-way ANOVA and post hoc Tukey test were applied for pairwise comparisons. Values with different letters within the same row are statistically different (P < 0.05).

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Figure 3. Peptidomics data. (A) Number of MS and MS/MS scans, number of identified peptides, 573 574 by MS/MS-data confrontation to Bos taurus protein database using PEAKS® Studio XPro, in caecal digesta 6h after ingestion of C1-, C2 low Ca²⁺-, CW2- or W-matrices (n = 8 rats/group). Means 575 576 without a common letter are significantly different (P < 0.05) after One-way ANOVA analysis followed by Tukey's multiple comparison test apart for the bottom left panel for which a Kruskall-577 578 Wallis test followed by a Dunn's multiple comparison test was used. Controls (n = 3) were excluded from the statistical analysis. Each individual point represent individual animals. (B) Heat 579 maps highlighting the occurrence of identified peptides along the amino acid sequences of aS1-580 581 casein and β -casein. A color was associated to each amino acid from white (no apparition 582 frequency) to red (high apparition frequency); more red color, more frequently detected peptides 583 on the backbone. (C) Multiple factor analysis (MFA) of α S1-casein- and β -casein-heat maps 584 constructed from identified peptides. The variables were divided into two dimensions (Dim 1 and 585 Dim 2). Individual factor map representing the repartition of each rat according to Dim 1 and Dim 586 2. The red circle represents rats with a low number of peptide identification.

587

Figure 4. Incorporation of dietary nitrogen to plasma proteins over the 6 h-postprandial period.
 Values are mean ± sem, n = 8 rats. The group- and protein matrix-effects were tested through

23

590 mixed linear models with repeated measures with * indicating a significant difference (P < 0.05) 591 between CW2 and C2 low Ca²⁺ while [#] indicating a significant difference (P < 0.05) between W and 592 the 3 other protein matrices.

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Figure 5. Fractional synthesis rate (FSR) of proteins in the liver (A) and gastrocnemius muscle (B) 6 h after the meal. Values are mean \pm sem, n = 8 rats /group. The effect of protein matrices was tested with a one-way ANOVA model and no significant diffence was observed (P > 0.05).

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Figure 6. Plasma concentrations of total amino acids (A), indispensable amino acids (B), and leucine (C). Values are expressed as mean \pm sem, n = 8 rats /group. The group and time effects were tested through mixed linear models with repeated measures. n.s, not significant. * indicates a significant difference (P < 0.05) between W and the groups C1 and C2 low Ca2+ within a time point. AA, amino acids.

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Figure 1













Α

Β

С

Differential effects of milk protein on amino acid digestibility, post-prandial nitrogen utilization and intestinal profile of peptides in rats

Nathalie Atallah¹²³, Claire Gaudichon¹, Audrey Boulier², Alain Baniel², Benoit Cudennec³, Barbara Deracinois³, Rozenn Ravallec³, Christophe Flahaut³, Dalila Azzout-Marniche¹, Nadezda Khodorova¹, Martin Chapelais¹, Juliane Calvez¹

¹UMR PNCA, AgroParisTech, INRAE, Université Paris-Saclay, 91123 Palaiseau, France

²Ingredia S.A. 62033 Arras Cedex, France

³UMRt BioEcoAgro-INRAe 1158, Univ. Lille, Univ. Artois, Institut Charles Viollette, 59000 Lille, France

	C1	C2 low Ca ²⁺	CW2	w
Dispensable amino acids				
Alanine	25	24	30	40
Arginine	39	40	37	31
Aspartate	59	56	71	94
Glutamate	190	184	173	157
Glycine	14	14	15	14
Proline	102	103	83	49
Serine	57	58	53	50
Tyrosine	55	58	49	38
Indispensable amino acids				
Histidine	63	65	60	49
Isoleucine	51	52	54	59
Leucine	89	90	101	120
Lysine	74	71	77	88
Methionine	28	28	25	22
Phenylalanine	49	50	47	41
Threonine	41	42	46	52
Valine	60	60	57	51

Supplementary table S1. Amino acid compositions (mg/g protein) of the 4 milk protein matrices

Amino acid composition were determined on 5 replicates of each milk protein matrix and molecular weight "in chain" were used.