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

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26 **Abstract**

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

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

34 **Results:** Orocaecal digestibility values of amino acids ranged between 96.0 ± 0.2% and 96.6 ± 0.4%
35 for C1-, C2 low Ca²⁺- and W-matrices, while this value was significantly lower for CW2 matrix (92.4
36 ± 0.5%). More dietary nitrogen was sequestered in the splanchnic area (intestinal mucosa and
37 liver) as well as in plasma proteins after ingestion of W matrix, especially compared to the C1-
38 and C2 low Ca²⁺-matrices. Peptidomic analysis showed that more milk protein-derived peptides
39 were identified in the caecum of rats after the ingestion of the matrices containing caseins
40 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

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

67 Some impacts of structural and physicochemical properties on the way that whey proteins and
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
73 stomach (Mahe et al., 1995). This fast digestion kinetics enhanced postprandial protein synthesis
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).

76 Evaluation of the protein quality implies its ability to satisfy the nitrogen and amino acid metabolic
77 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
80 et al., 2021; Deglaire, Moughan, Airinei, Benamouzig, & Tome, 2020; Gaudichon et al., 2002).
81 However, less is known about how the industrial processes affect digestibility and postprandial
82 utilization of milk proteins. A rat study, dedicated to impacts of milk heat treatments
83 demonstrated that the protein quality of microfiltered milk was maintained after different types
84 of heating, except for spray drying which decreased its digestibility (Lacroix, Leonil, et al., 2006).
85 Moreover, the type of applied processing modifies the protein structure,, notably for casein
86 micelles that undergo demineralization. This demineralization process is achieved through pH
87 changes (acidification or alkalization) (Silva et al., 2013) or phosphorus and calcium chelation (de
88 Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2011). The acidification process, followed
89 by neutralization of the curd, results in sodium caseinates where the casein micelles have an
90 irregular shape and porous structure (Coskun, Sağlam, Venema, van der Linden, & Scholten, 2015)
91 compared to those obtained by membrane filtration that preserves their integrity (Dalgleish &
92 Corredig, 2012b). An *in vitro* study using a dynamic digestion model has shown that native
93 micellar caseins and sodium caseinates were digested differently (Wang, Ye, Lin, Han, & Singh,
94 2018). Indeed, gastric hydrolysis of resolubilized milk powder containing the native form of
95 caseins was much slower than for sodium caseinates and that was related to its better coagulation
96 ability. Moreover, a static *in vitro* study has shown that demineralized and reticulated sodium
97 caseinates generated a different peptide pattern compared to native micellar caseins, suggesting
98 an effect of protein structure on its digestion (Atallah et al., 2020).

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

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
111 g of ¹⁵N ammonium sulfate [99%, (¹⁵NH₄)₂SO₄] twice a day for a period of 9 days. Collection of the
112 milk occurred from the fourth to the ninth day. An isotopic enrichment of 1.4 atom percent in the
113 milk was obtained. The ¹⁵N-labeled milk was then pasteurized (75°C, 15 s) prior to protein
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
116 concentrated in native micellar caseins (C1 and C2 low Ca²⁺) with C2 low Ca²⁺ having the lowest
117 calcium concentration. The CW2 matrix contained a lower casein-to-whey protein ratio (67:33)
118 compared to the one occurring naturally in cow milk (80:20) and the W matrix contained only
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
134 were subjected to an adaptation protocol for 1 week and randomly split into 4 groups (n =

135 8/group) according to the milk protein matrices they would consume: native micellar caseins (C1),
136 native micellar casein low in calcium (C2 low Ca²⁺), casein and whey proteins in a 67:33 ratio
137 (CW2) and whey (W). The objective of this adaptation protocol (Figure 1B) was to get the rats
138 used to consuming a single meal in a limited time-frame as described previously (Atallah et al.,
139 2022; Guillin et al., 2021; Tessier et al., 2020). This latter involved giving them access to a first
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
144 unlimited access to water during the whole experimental protocol.

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

160

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

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

164 determination of the nitrogen percentage and ¹⁵N enrichment in the digestive contents and meals
165 was performed with an elemental analyzer (Vario Micro Cube, Elementar, Langenselbold,
166 Germany) coupled with isotopic ratio mass spectrometry (IsoPrime, GV Instruments Ltd.,
167 Manchester, UK; EA-IRMS). Determination of the ¹⁵N-enrichment in each amino acid of digesta
168 and meals was obtained with gas chromatography (GC 6890 N, Agilent Technologies, Les Ulis,
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
172 method (Waters, France), as previously described (Guillin et al., 2021).

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

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

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

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

$$184 \quad \text{True nitrogen oro-caecal digestibility} = 100 \times \frac{N_{\text{ing}} - (N_{\text{diet ileum}} + N_{\text{diet caecum}})}{N_{\text{ing}}}$$

185 Where N_{ing} is the amount of nitrogen ingested by the rats (mmol). True oro-caecal nitrogen
186 digestibility was also determined considering nitrogen losses in the colon and feces, in addition
187 to losses in the ileum and caecum. Similarly to true oro-caecal nitrogen digestibility, true oro-caecal
188 digestibility for each individual amino acid was determined by estimating the dietary amino acids
189 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,
193 Randox, Crumlin, UK). Plasma was separated in protein-, free amino acid- and urea-fractions as
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
197 was a proxy of the urinary urea ¹⁵N-enrichment and calculated the dietary nitrogen amount
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
201 amount recovered in proteins of organs or plasma (mmol) was calculated as described previously
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
204 weight for plasma protein calculation. (Waynforth & Flecknell, 1992).

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

208

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

210 *In vivo* fractional synthesis rates (FSR) of proteins in the gastrocnemius muscle and liver were
211 determined using the [¹³C]-valine flooding dose method as previously described (Chevalier et al.,
212 2013; Oberli et al., 2015). Briefly, the protein-bound and free amino acid fractions were separated
213 in each tissue. [¹³C]-valine enrichment in the free amino acid fraction was estimated by gas
214 chromatography (GC 6890N, Agilent Technologies) coupled to mass spectrometry (MS 5973N,
215 Agilent Technologies) (GC-MS). Enrichment in [¹³C]-valine in protein bound amino acid fraction

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

218

219 **2.5. Measurement of plasma amino acid kinetics**

220 Plasma concentration of free amino acids, 0, 1, 3 and 6 h after meal ingestion, was determined,
221 after protein precipitation, by UHPLC with AccQTag Ultra method (Waters) as previously
222 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
229 concentration of 10 mg.mL⁻¹ in water containing 0.1% trifluoroacetic acid (TFA) and centrifuged.
230 Peptides were concentrated ten times and desalted with C18 solid phase extraction (SPE) columns
231 (Bond Elut C18 1000 mg minicolumns, Agilent Technologies). Dried peptides were dissolved in
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),
238 (v/v)). The ACN gradient (flow rate 0.5 mL.min⁻¹) was as follows: from 1% to 40% solvent B over
239 22.5 min, from 40% to 95% solvent B over 2.5 min followed by washing and equilibrating
240 procedures with 95% and 1% solvent B for 2.5 min each, respectively. The eluate was injected
241 into the electrospray ionization source of a qTOF Synapt G2-Si™ (Waters). Database searches
242 were performed using the UniProt database restricted to *Bos taurus* organism via PEAKS® Studio

243 XPro software (Bioinformatics Solutions Inc., Waterloo, Canada). The peptide identity searches
244 were performed without notifying the choice of enzyme.

245

246 **2.7. Statistical analyses**

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

253 All results are expressed as mean \pm 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
256 were applied for pairwise comparisons. Mixed linear models with repeated measures were
257 performed to test differences between groups over time. Differences were considered
258 statistically significant with a p-value < 0.05. For peptidomic analysis, a multiple factor analysis
259 (MFA) was conducted to comparing data from MS analysis (α S1-casein and β -casein heat maps).
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
262 package (Lê, Josse, & Husson, 2008).

263

264 **3. Results**

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

266 No difference in body weight gain and food intake (data not shown) was observed between the
267 4 groups of rats. The final body weights were 291.8 ± 5.8 g, 291.8 ± 5.0 g, 288.0 ± 5.8 g and 290.3
268 ± 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**

271 **Figure 2** represents the quantity of dietary nitrogen collected in the different portions of the
272 gastrointestinal tract according to the milk protein matrix ingested by the rats. Whatever the
273 matrix, the majority of the dietary nitrogen was recovered in the caecum of rats (Figure 2).
274 Therefore, in the caecum, a significant effect of the matrix was observed ($P = 0.0046$) with a
275 significantly higher amount of dietary nitrogen after ingestion of CW2-matrix in comparison to C1
276 ($P = 0.0377$), C2 low Ca^{2+} ($P = 0.0032$) and W ($P = 0.0363$). No significant difference between the
277 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 Ca^{2+}) for oro-caecal digestibility. Oro-caecal
283 nitrogen digestibility was significantly lower for the CW2 matrix in comparison to C1 ($P = 0.0405$),
284 C2 low Ca^{2+} ($P = 0.0016$) and W ($P = 0.0094$). The same tendency was observed with the oro-faecal
285 nitrogen digestibility. Regarding individual amino acid digestibility values, their average oro-caecal
286 digestibility varied from $92.4 \pm 0.5\%$ (for CW2) to $96.6 \pm 0.4\%$ (for C1), and the same tendency
287 was observed for the CW2 matrix, which had a significantly lower value compared to the other
288 matrices ($P < 0.0001$). Indeed, the oro-caecal digestibility was lower for the CW2 matrix for most
289 of the individual amino acids except for methionine, alanine, glycine, and serine.

290

291 **3.4. Peptide identification in the caecum**

292 **Figure 3** gathers the different data obtained by peptidomics analysis of the caecal digesta 6h after
293 ingestion of C1-, C2 low Ca^{2+} -, CW2- or W-matrices ($n = 8$ rats/group).

294 As illustrated in Figure 3A (upper panels), whatever the milk protein matrix used, the average
295 number of MS- and MS/MS-scans recorded during the HPLC-MS/MS runs were $2,091 \pm 340$ MS
296 scans and $6,452 \pm 675$ 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
298 differences between groups. In contrast, the number of peptides identified by MS/MS-data
299 confrontation to *Bos taurus* protein database using PEAKS® Studio XPro (Figure 3A (bottom
300 panels)) were statistically different as highlighted by the lowercase letters. Indeed, the number
301 of identified peptides was significantly more important from caecum samples corresponding to
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 ±
305 6, respectively. The full list of all identified casein peptides for each experimental condition is
306 presented in supplementary Table S2. Interestingly, no peptide from kappa-casein nor whey
307 proteins were identified from the 8 biological replicates whatever the milk protein matrix used.
308 Moreover, as expected, the few peptides identified from the caecum samples of control rats were
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.

312 Heat maps (Fig. 3B), displaying the amino acid occurrences in αS1- and β-casein according to the
313 ingestion of C1-, C2 low Ca²⁺-, CW2- or W-matrices, show the protein-backbone zones which were
314 resistant to gastrointestinal digestion and intestinal absorption. For αS1-casein, except for the W-
315 matrix where no casein peptides were identified, the same resistant zones were found after
316 ingestion of C1-, C2 low Ca²⁺- and CW2-matrices. The resistant zones were distributed over the
317 N-terminal half of the protein skeleton. For β-casein, overall, only one main N-terminal zone
318 resistant to gastrointestinal digestion emerges for all ingested matrices.

319 The 2D-plot graph corresponding to the first two dimensions issued from the MFA, constructed
320 from all identified αS1- and β-casein peptides of each individual (Fig. 3C), highlights the similarity
321 and discrepancy between the samples. The green plots related to the ingestion of whey proteins
322 were distinctly different from the others, except for one (over the 8) of each of the 3 casein
323 matrices. Moreover, whatever the ingested-casein matrix, no set of individuals was different from
324 the two others.

325

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

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

345

346 **3.6. Postprandial incorporation of dietary nitrogen to plasma protein**

347 The incorporation of dietary nitrogen to plasma proteins was evaluated according to the different
348 matrices over the 6 h-postprandial period (**Figure 4**). Significant effects of groups, time and their
349 interaction ($P < 0.0001$) were observed during the whole postprandial period. Starting from 3 h
350 after ingestion, the W matrix started to differentiate from the other matrices as more dietary
351 nitrogen was incorporated to plasma proteins compared to CW2 ($P = 0.0094$), and both C1 and
352 C2 low Ca^{2+} ($P < 0.0001$). Interestingly, 6 h after ingestion, the W matrix still showed the highest

353 incorporation of dietary nitrogen compared to other matrices ($aP < 0.0001$ for both, CW2, C1 and
354 C2 low Ca^{2+}). The CW2 matrix also incorporated significantly more dietary nitrogen into plasma
355 protein compared to the C2 low Ca^{2+} 3 h and 6 h after meal ingestion ($P = 0.0009$ and $P = 0.0029$,
356 respectively). During the entire postprandial period, no difference between the casein-based
357 matrices, C1 and C2 low Ca^{2+} , were observed.

358

359 **3.7. *In vivo* protein synthesis**

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

363

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
368 as leucine ($P < 0.0001$) concentration kinetics, with an increase in amino acid concentrations after
369 meal ingestion and a return to basal level at 6 h. The casein-based matrices (C1 and C2 low Ca^{2+})
370 exhibited a prolonged plateau over 6 h after meal ingestion, however no significant effect of
371 groups was observed regarding kinetics of total- or indispensable-amino acid concentrations. In
372 contrast, plasma leucine concentration kinetics was influenced by groups (group x time effect: P
373 < 0.0001) and ingestion of the W matrix induced a significantly higher increase of leucine
374 concentration 1 h after meal intake in comparison to C1 ($P = 0.0021$) and C2 low Ca^{2+} ($P = 0.0034$).

375

376 **4. Discussion**

377 The aim of this study was to characterize the digestibility and postprandial utilization of ^{15}N -
378 labeled milk protein matrices, obtained by different industrial processes, *in vivo*. The matrices

379 included two micellar casein-based matrices with varying calcium concentrations (C1 and C2 low
380 Ca^{2+}), 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
383 protein isolate exhibited ileal nitrogen digestibility from 91 to 95% and mean amino acid ileal
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%,
386 depending on the protein matrix, with higher values for whey (98 - 100%) (Lacroix, Leonil, et al.,
387 2006; Rutherfurd, Fanning, Miller, & Moughan, 2015). Our results are partly in accordance with
388 these previously published studies as the nitrogen oro-caecal digestibility (a proxy of ileal
389 digestibility) of the casein-based matrices were 94.9% and 95.5% for native caseins (C1) and
390 caseins with low calcium (C2 low Ca^{2+}), 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 oro-caecal
393 digestibility, respectively) in comparison to previously published data (Lacroix, Leonil, et al., 2006;
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 oro-caecal 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 oro-caecal 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
403 between whey proteins and caseins which resulted in structural changes that limited the
404 accessibility to digestive enzymes in the CW2 matrix. Such an hypothesis is supported by the
405 higher amount of free thiol groups available from β -lactoglobulin which can lead to an increase
406 in the formation of disulfide bonds between caseins and whey proteins (Zhao et al., 2016).

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
409 caecum contents contained milk protein-derived peptides which belong to the same parent
410 proteins. Since the number of identified peptides was higher in the caecal digesta, we decided to
411 deeply analyse the results obtained for them. The non-statistically different number of MS- and
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
415 are fully comparable (Atallah et al., 2020). First of all, the overall number of peptides identified
416 from caecal content ranges from 50 ± 35 to 6 ± 5 according to the milk matrix ingested, and the
417 number of identified peptides was significantly lower in the caecal digesta for the whey matrix
418 compared to matrices containing mainly caseins. Recently, Sanchón *et al.* reported, the
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).
425 In our study, no peptides from the whey proteins were identified in the caecum whatever the
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
435 XPro). This latter point agrees with the results reported by Sanchón *et al.* (Sanchon et al., 2018).

436 Finally, the protein backbone of α 1-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
440 digested in contrast to whey proteins that are rapidly digested. This has been demonstrated
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
443 ingestion in comparison to casein in humans (Lacroix, Bos, et al., 2006). We did not clearly observe
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.
447 However, the rats that consumed the whey protein (W matrix) meal exhibited a plasma leucine
448 concentration increase 1 h after ingestion in comparison to the two other casein-based matrices
449 (C1 en C2 low Ca^{2+}), whereas no difference was observed with the CW2 matrix. The whey protein
450 matrix presents the highest leucine content so it triggers a more massive leucine appearance
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
453 digestibility resulted in a slight plateau-type increase of plasma leucine whereas no increase was
454 observed for the casein matrices.

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

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
469 of milk proteins ranging from 8 to 11% and no difference between the milk protein matrices.
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
473 kinetics of dietary nitrogen transfer to urea, a difference might have been observed, since transfer
474 of dietary nitrogen to urea was quicker for whey proteins and reached a maximum 2 h after
475 ingestion compared to caseins or total milk proteins, as reported in a human clinical trial (Lacroix,
476 Bos, et al., 2006).

477 Despite higher incorporation of dietary nitrogen in muscle and liver, and a peak of plasma leucine
478 concentration after whey proteins ingestion, no difference in terms of protein synthesis was
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;
482 Kanda et al., 2016; Tessier et al., 2019). Similarly, no difference in muscle and liver FSR of proteins
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,
485 & Mosoni, 2011; Adechian et al., 2012). In humans, the effects of different milk proteins on the
486 stimulation of muscle protein synthesis seemed to be highly dependent on the structure of the
487 matrix and the composition in amino acids of the milk proteins (Reitelseder et al., 2011; Tang et
488 al., 2009; Tipton et al., 2004). However, differences could have been pointed out if the FSR of
489 proteins had been measured at different time points. Indeed, in a study evaluating protein
490 anabolism after different milk protein consumption in rats, the muscle protein FSR measured 4 h
491 after ingestion was similar after the whey protein, the caseinate and the milk protein intake, but
492 time-course of FSR time varied among the different protein types (Kanda et al., 2016).

493

494 **5. Conclusions**

495 All the milk protein matrices tested in this study had high digestibility values. However, our results
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
499 postprandial metabolism was observed after ingestion of micellar caseins and caseins with low
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
502 and whey matrices, a higher incorporation of dietary nitrogen in most tissues was found, in
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 1-, α 2- and β -caseins and are mainly located in the
506 N-terminal half of the α 1-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
508 processes influence protein digestion and utilization. Knowledge of the consequences of
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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524

525 **Conflict of interest**

526 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.
527 N.A., A.Ba., and A.Bo. are employed by Ingredia.

528

529 **Authorship**

530 The authors’ responsibilities were as follows: J.C., C.G., A.Ba. and A.Bo. contributed to the
531 conception and design of the study; N.A., J.C. conducted the research; N.A., J.C., N.K., M.C.,
532 contributed to sample analyses, N.A. and J.C. analyzed the data; B.C, B.D. C.F. and R.R conducted
533 the peptidomic analysis of the digestive contents; N.A. wrote the original draft of the paper; all
534 authors reviewed and edited the manuscript; C.G. administered the project, and all authors read
535 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**

541 **Table 1. Characteristics of the milk protein matrices**

| | Native micellar casein (%) | Whey protein (%) | Calcium (%) | Total protein content (%) |
|-------------------------------|----------------------------|------------------|-------------|---------------------------|
| C1 | 92 | 8 | 2.6 | 83 |
| C2 low Ca²⁺ | 92 | 8 | 1.6 | 85 |
| CW2 | 63 | 37 | 1.8 | 81 |
| W | < 1 | > 99 | 0.3 | 81 |

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 oro-caecal digestibility (%) | | | | | |
| Isoleucine | 96.0 ± 0.5 ^a | 95.5 ± 0.3 ^a | 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 ± 0.3 ^a | 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 ± 0.3 ^a | 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 ± 0.2 ^a | < 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 oro-caecal 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 ± 0.3 ^a | 95.8 ± 0.3 ^a | 90.0 ± 0.6 ^b | 97.2 ± 0.3 ^a | < 0.0001 |
| Glycine | 97.4 ± 0.4 | 97.4 ± 0.3 | 96.7 ± 0.3 | 97.5 ± 0.2 | <i>n.s.</i> |
| Glutamate + glutamine | 96.0 ± 0.6 ^a | 94.7 ± 0.5 ^a | 87.9 ± 0.8 ^b | 97.0 ± 0.5 ^a | < 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 | <i>n.s.</i> |
| 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 digestibility¹ (%) | 96.6 ± 0.3 ^a | 96.0 ± 0.2 ^a | 92.4 ± 0.5 ^b | 96.6 ± 0.4 ^a | < 0.0001 |
| Nitrogen digestibility (%) | | | | | |
| Oro-caecal digestibility | 94.9 ± 0.4 ^a | 95.5 ± 0.3 ^a | 93.5 ± 0.3 ^b | 95.2 ± 0.3 ^a | 0.0019 |
| Oro-faecal digestibility | 93.9 ± 0.4 ^a | 94.4 ± 0.3 ^a | 92.1 ± 0.4 ^b | 94.2 ± 0.3 ^a | 0.0004 |

552 Values are mean ± sem, n = 8 rats/group. ¹Average digestibility was calculated from the mean amino acid
553 digestibilities weighted by the proportion of each amino acid in the milk protein matrix. The effect of the protein
554 matrix was tested with a one-way ANOVA model. Values with different letters within the same row are statistically
555 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 Ca²⁺ | CW2 | W | P value |
|-----------------------------------|--------------------------|-------------------------------|--------------------------|--------------------------|----------------|
| Body urea pool | 2.5 ± 0.4 | 2.6 ± 0.5 | 3.2 ± 0.7 | 2.5 ± 0.5 | <i>n.s.</i> |
| Urinary urea pool | 5.9 ± 0.8 | 5.2 ± 0.7 | 8.1 ± 1.3 | 6.7 ± 0.5 | <i>n.s.</i> |
| Total deamination | 8.4 ± 0.9 | 7.8 ± 0.8 | 11.3 ± 1.7 | 9.2 ± 0.7 | <i>n.s.</i> |
| Liver ¹ | 12.6 ± 0.7 ^a | 11.4 ± 0.5 ^a | 14.8 ± 1.4 ^a | 19.5 ± 0.7 ^b | < 0.0001 |
| Gastrocnemius muscle ¹ | 0.50 ± 0.03 ^a | 0.41 ± 0.02 ^a | 0.51 ± 0.04 ^a | 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 | <i>n.s.</i> |
| Jejunum ² | 0.27 ± 0.02 ^a | 0.26 ± 0.01 ^a | 0.35 ± 0.02 ^b | 0.42 ± 0.02 ^c | < 0.0001 |
| Ileum 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**

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

566
567 **Figure 2. Dietary nitrogen (expressed as % of ingested nitrogen) recovered in gastrointestinal**
568 **tract contents according to the milk protein matrix.** Values are mean \pm sem, n = 8 rats/group.
569 The group effect was tested using a one-way ANOVA and post hoc Tukey test were applied for
570 pairwise comparisons. Values with different letters within the same row are statistically different
571 (P < 0.05).

572
573 **Figure 3. Peptidomics data. (A)** Number of MS and MS/MS scans, number of identified peptides,
574 by MS/MS-data confrontation to *Bos taurus* protein database using PEAKS® Studio XPro, in caecal
575 digesta 6h after ingestion of C1-, C2 low Ca²⁺-, CW2- or W-matrices (n = 8 rats/group). Means
576 without a common letter are significantly different (P < 0.05) after One-way ANOVA analysis
577 followed by Tukey's multiple comparison test apart for the bottom left panel for which a Kruskal-
578 Wallis test followed by a Dunn's multiple comparison test was used. Controls (n = 3) were
579 excluded from the statistical analysis. Each individual point represent individual animals. **(B)** Heat
580 maps highlighting the occurrence of identified peptides along the amino acid sequences of α S1-
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
588 **Figure 4. Incorporation of dietary nitrogen to plasma proteins over the 6 h-postprandial period.**
589 Values are mean \pm sem, n = 8 rats. The group- and protein matrix-effects were tested through

590 mixed linear models with repeated measures with * indicating a significant difference ($P < 0.05$)
591 between CW2 and C2 low Ca^{2+} while # indicating a significant difference ($P < 0.05$) between W and
592 the 3 other protein matrices.

593
594 **Figure 5. Fractional synthesis rate (FSR) of proteins in the liver (A) and gastrocnemius muscle**
595 **(B) 6 h after the meal.** Values are mean \pm sem, $n = 8$ rats /group. The effect of protein matrices
596 was tested with a one-way ANOVA model and no significant difference was observed ($P > 0.05$).

597
598 **Figure 6. Plasma concentrations of total amino acids (A), indispensable amino acids (B), and**
599 **leucine (C).** Values are expressed as mean \pm sem, $n = 8$ rats /group. The group and time effects
600 were tested through mixed linear models with repeated measures. n.s, not significant. * indicates
601 a significant difference ($P < 0.05$) between W and the groups C1 and C2 low Ca^{2+} within a time
602 point. AA, amino acids.

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740

Figure 1

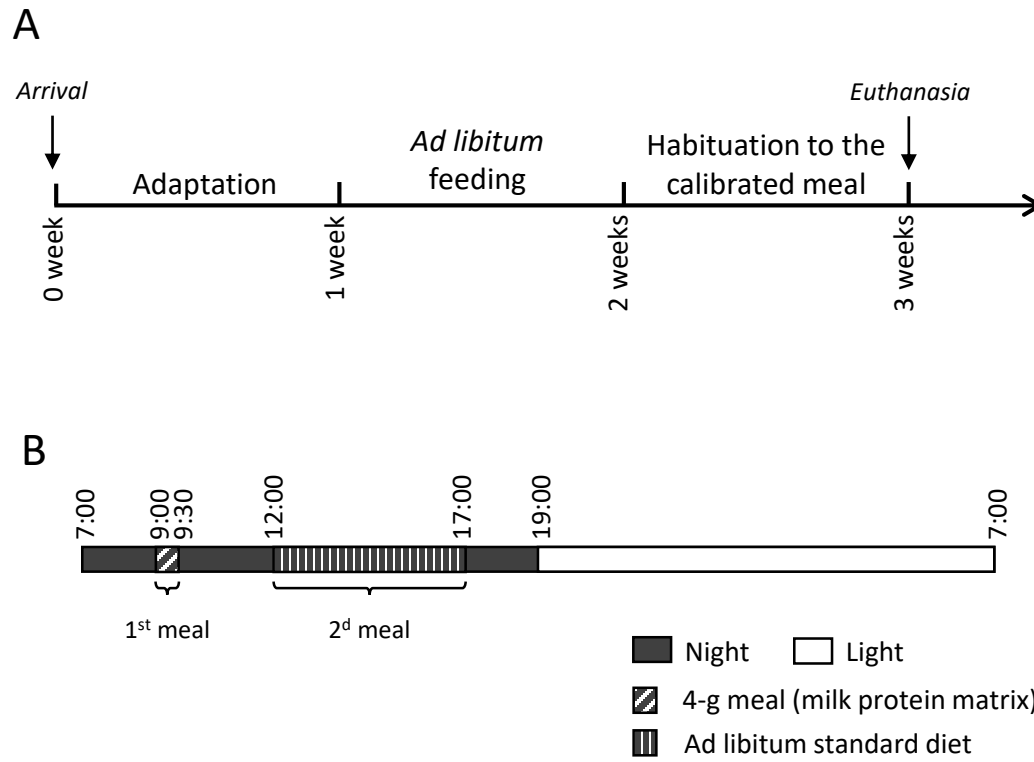


Figure 2

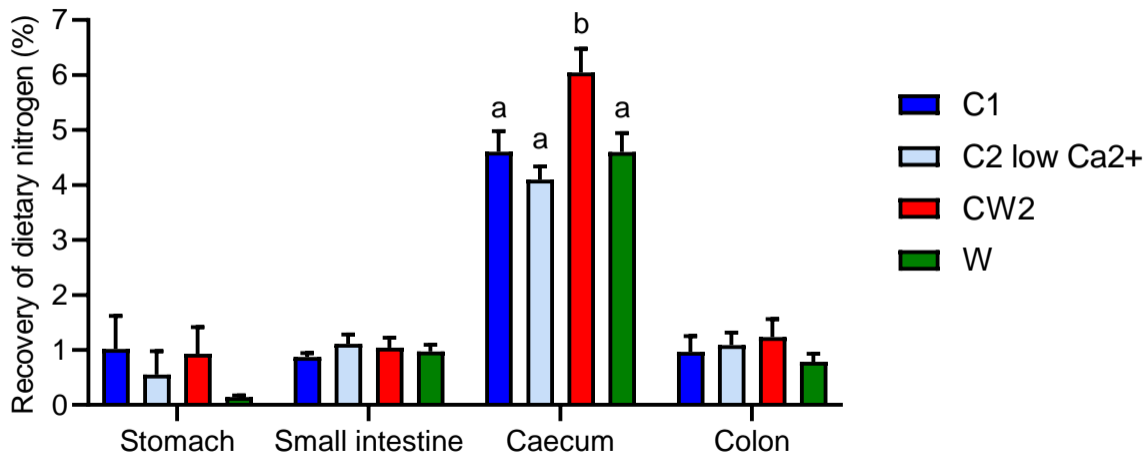
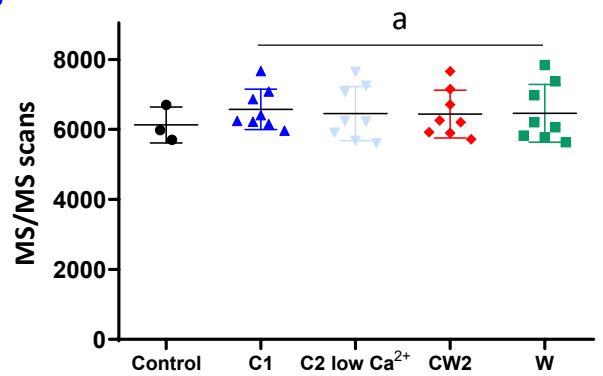
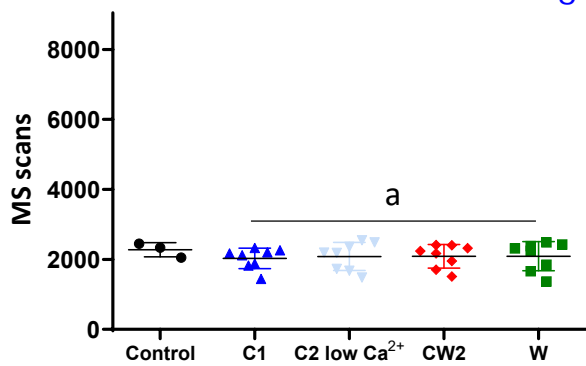
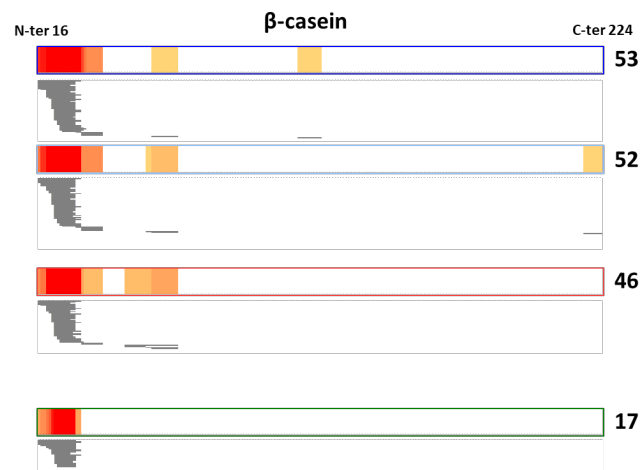
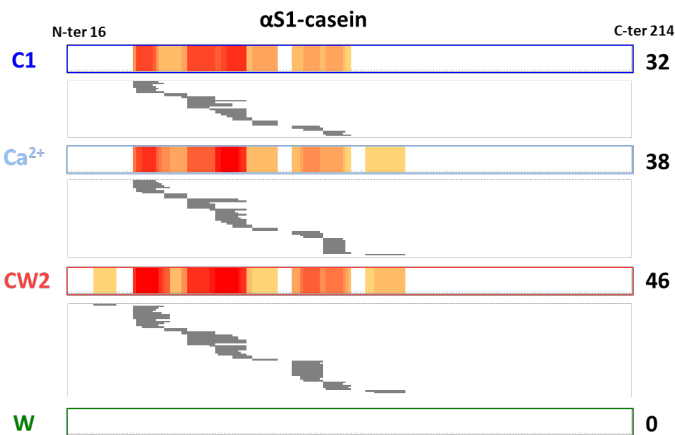
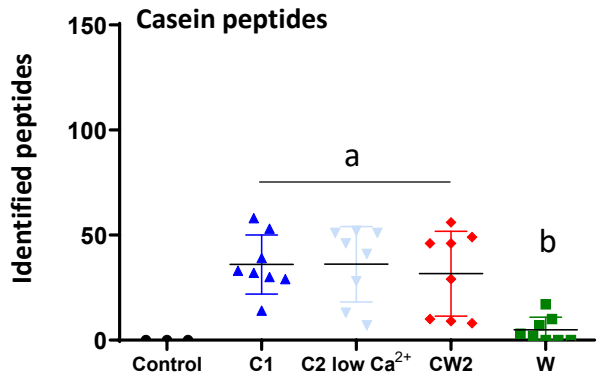
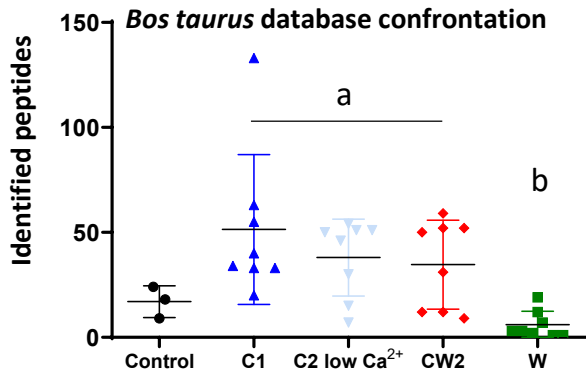


Figure 3

A



B



C

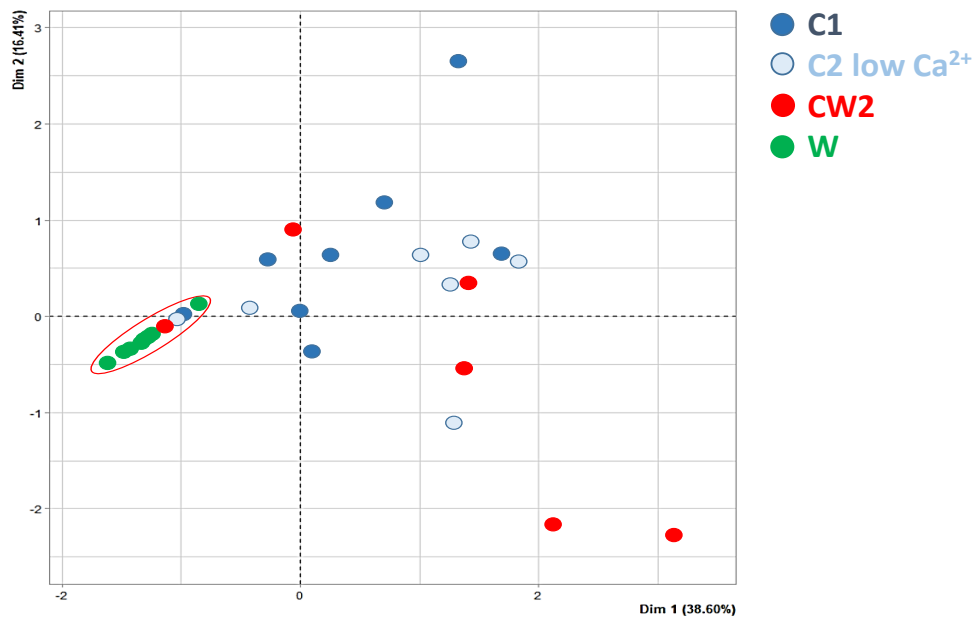


Figure 4

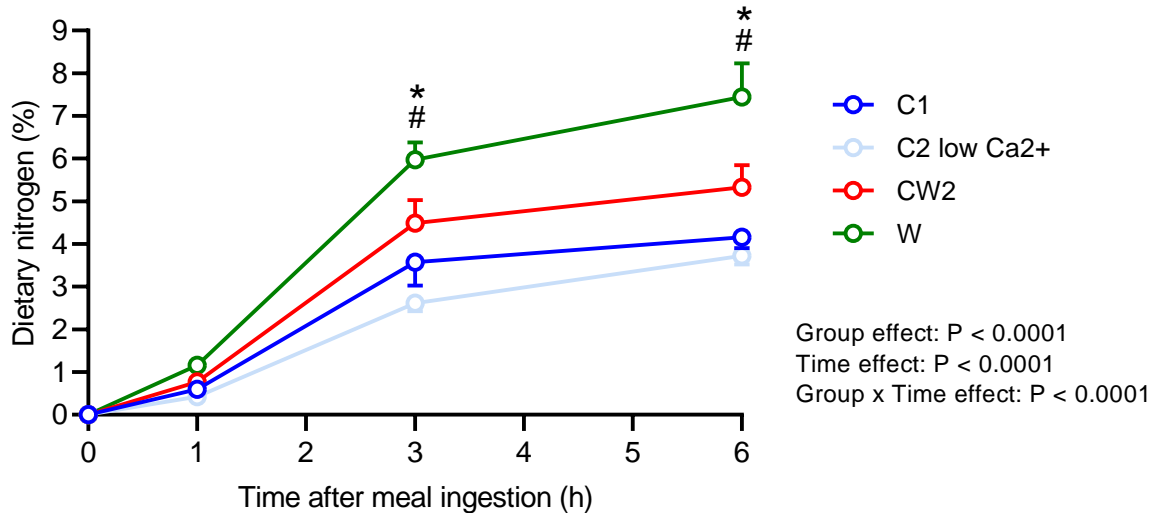
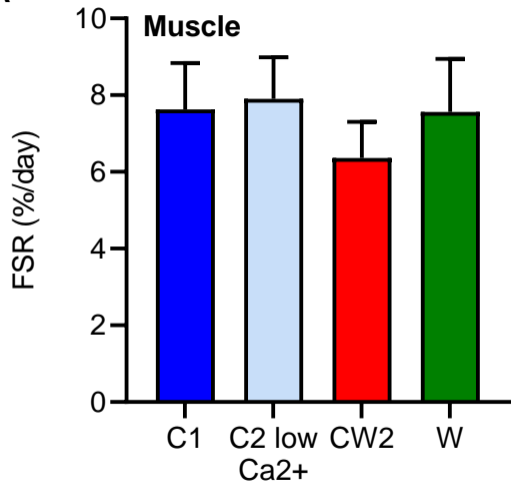


Figure 5

A



B

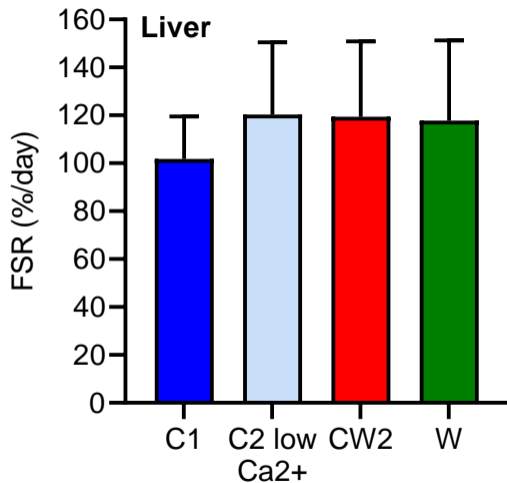
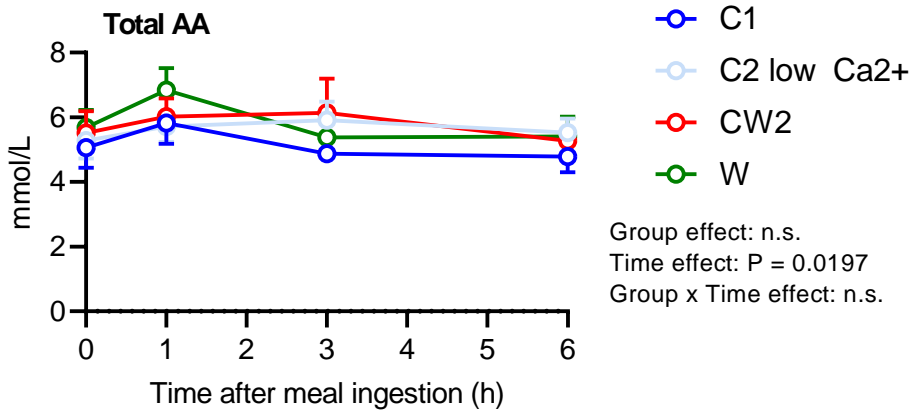
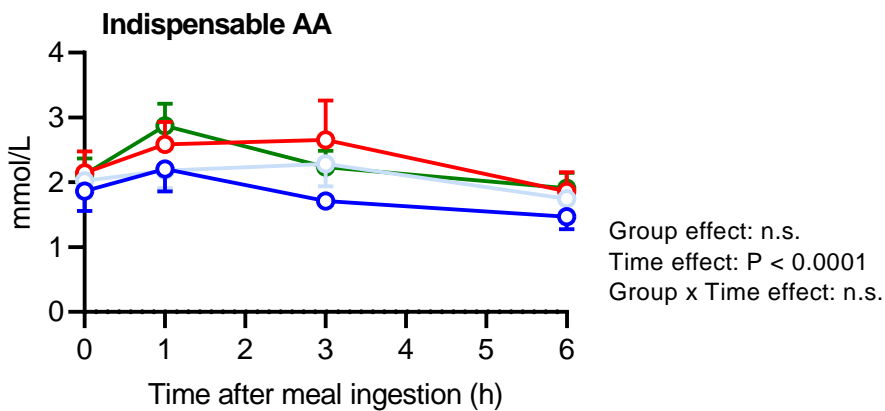


Figure 6

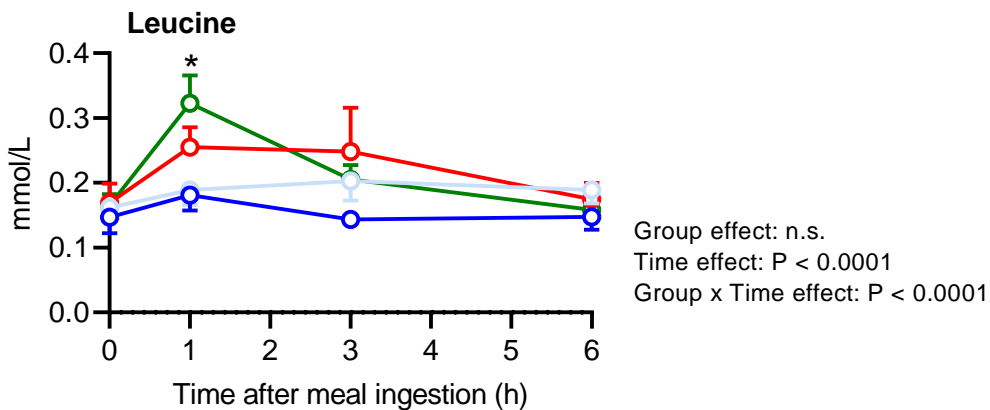
A



B



C



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

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

| | C1 | C2 low Ca²⁺ | 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 |

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