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Nathalie Atallah[123], Claire Gaudichon[1], Audrey Boulier[2], Alain Baniel[2], Benoit Cudennec[3], Barbara Deracinois[3], Rozenn Ravallec[3], Christophe Flahaut[3], Dalila Azzout-Marniche[1], Nadezda Khodorova[1], Martin Chapelais[1], Juliane Calvez[1]*

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

[2]Ingredia S.A. 62033 Arras Cedex, France

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

*** Corresponding author**

Juliane Calvez. AgroParisTech-INRAE, UMR 914 PNCA, 22 place de l'agronomie, 91123 Palaiseau, France. Email: juliane.calvez@agroparistech.fr

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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.

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 ^{15}N-labeled milk proteins. Four milk matrices were tested: native micellar caseins (C1), caseins low in calcium (C2 low Ca^{2+}), 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 ± 0.2% and 96.6 ± 0.4% for C1-, C2 low Ca^{2+}- and W-matrices, while this value was significantly lower for CW2 matrix (92.4 ± 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 C1- and C2 low Ca^{2+}-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.

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

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

1. Introduction

In the past five decades, milk protein extraction has been widely applied at the dairy industry level in order to use these proteins as food ingredients (Fox, 2001). Milk proteins are categorized into two classes: caseins which represent 80% of the protein content in cow milk and whey 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. Whey proteins have a well-established globular structure and include β-lactoglobulin, α-lactalbumin and other minor fractions. Caseins have a flexible structure and exist in four types that are more or less phosphorylated and glycosylated: α_{S1}-, α_{S2}-, β- and κ-caseins. The particularity of caseins is their ability to interact together and with calcium phosphate to form macromolecular aggregates named casein micelles. In contrast to whey proteins, the precise structure of the casein micelle is still a subject of debate and numerous theories exist (Fox & 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 surface of the micelle, providing it stability and limiting its growth. It is also well-established that calcium phosphate is necessary to maintain the integrity of the micelle (Dalgleish & Corredig, 2012a).

Some impacts of structural and physicochemical properties on the way that whey proteins and caseins are digested have already been well demonstrated. Indeed, the notion of “slow” and “fast” proteins was conceptualized when it was shown that caseins were digested in up to 6 h and whey proteins in 2 h (Boirie et al., 1997; Mahe et al., 1996). This was explained by the ability of caseins to form curds by acidic precipitation in the stomach, which delayed gastric emptying, 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 (Boirie et al., 1997; Burd et al., 2012; Dangin et al., 2001; Tang, Moore, Kujbida, Tarnopolsky, & 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
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 ^{15}N-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.

2. Material and methods

2.1. Labeling and production of the milk protein matrices

The milk was labeled by Ingredia (Arras, France) by providing two cows with water containing 50 g of ^{15}N ammonium sulfate [99%, $(^{15}NH_4)_2SO_4$] twice a day for a period of 9 days. Collection of the milk occurred from the fourth to the ninth day. An isotopic enrichment of 1.4 atom percent in the milk was obtained. The ^{15}N-labeled milk was then pasteurized (75°C, 15 s) prior to protein extraction, and a total of four protein matrices (**Table 1**) were obtained by filtration then 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 calcium concentration. The CW2 matrix contained a lower casein-to-whey protein ratio (67:33) compared to the one occurring naturally in cow milk (80:20) and the W matrix contained only whey proteins. The amino acid composition of the four matrices is presented in **supplemental Table S1**.

2.2. Animals and experimental design

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

8/group) according to the milk protein matrices they would consume: native micellar caseins (C1), native micellar casein low in calcium (C2 low Ca^{2+}), casein and whey proteins in a 67:33 ratio (CW2) and whey (W). The objective of this adaptation protocol (Figure 1B) was to get the rats 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 meal (weighing 4 g in dry weight) that had the same composition as the standard diet but that contained the respective protein matrices in isonitrogenous amounts. They had access to this first meal for 30 min (from 09:00 to 09:30) then to the standard diet *ad libitum* for 7 h (from 10:30 to 17:30). The rats were weighed every weekday, temperature was controlled and they had unlimited access to water during the whole experimental protocol.

On the day of euthanasia, the rats consumed the calibrated meals containing their respective ^{15}N-labeled milk protein matrices. Blood was collected from the tail vein in the fasting state as well as 1 h and 3 h after meal ingestion, and plasma was then stored at -20°C. Thirty minutes before euthanasia, the rats received an injection at the lateral vein of the tail of [1-^{13}C] valine (Eurisotop, Saint Aubin, France) at 150 µmol/100 g of body weight under gaseous anesthesia. Rats were euthanazied 6 h after meal ingestion, after cardiac puncture (to collect plasma) under gaseous 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 with NaCl (9%). Feces were gathered over the 6 h postprandial period. The contents were weighed 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 samples were also collected, frozen in liquid nitrogen and stored at -80°C. Absorbent papers were placed under the cages in order to retrieve urine during the 6 h postprandial period and the 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.

2.3. Measurement of nitrogen and amino acid digestibility

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

164 determination of the nitrogen percentage and ^{15}N 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 ^{15}N-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 $$\mathrm{N_{diet\ digesta}} = \mathrm{N_{tot}} \times \frac{\mathrm{APE_{digesta}}}{\mathrm{APE_{meal}}}$$

176 Where N_{tot} represents the total amount of nitrogen in the sample (mmol) and APE the enrichment
177 excess of ^{15}N 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 orocaecal nitrogen digestibility (%) was calculated as follows:

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

185 Where N_{ing} is the amount of nitrogen ingested by the rats (mmol). True orofecal 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 orocaecal nitrogen digestibility, true orocaecal
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.

2.4. Measurement of nitrogen postprandial utilization

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 described previously (Bos et al., 2005). Urea was assumed to be uniformly distributed throughout the total body water and dietary nitrogen amount recovered in the urea body pool (mmol) as described previously (Lacroix, Leonil, et al., 2006). We assumed that the urinary ^{15}N-enrichment was a proxy of the urinary urea ^{15}N-enrichment and calculated the dietary nitrogen amount recovered in the urinary as described previously (Lacroix, Leonil, et al., 2006). The total amount of dietary nitrogen transferred to urea was obtained from the sum of the dietary nitrogen amount 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 (Tessier et al., 2019). The dietary nitrogen amount recovered in the different tissues was 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).

The ^{15}N-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.

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 [^{13}C]-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. [^{13}C]-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 [^{13}C]-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).

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).

2.6. Analysis of peptidomic profil in cecal digesta

For peptidomics analysis, digestive samples obtained from rats (n = 3) fed a protein-free meal in a study with comparable design (Guillin et al., 2021) was included as control. Peptide sequence identifications in the caecal digesta samples were performed as previously described with some 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. Peptides were concentrated ten times and desalted with C18 solid phase extraction (SPE) columns (Bond Elut C18 1000 mg minicolumns, Agilent Technologies). Dried peptides were dissolved in ultra-pure H_2O containing 0.1% TFA (v/v), centrifuged and supernatants were analyzed in triplicate by reverse phase-high performance liquid chromatography coupled to tandem mass spectrometry (RP-HPLC-MS/MS). Peptides were chromatographically separated at 30°C on an Acquity UPLC system (Waters Corporation, France) using a C18AQ column (150 × 3.0 mm, 2.6 µm, Uptisphere CS EVOLUTION, Interchim, France). The mobile phases consisted of solvent A (0.1% 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 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 into the electrospray ionization source of a qTOF Synapt G2-Si™ (Waters). Database searches 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 ± 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 (αS1-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 Ca^{2+}-, CW2- and W-milk protein matrix, respectively.

269

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 Ca^{2+} ($P = 0.0032$) and W ($P = 0.0363$). No significant difference between the milk protein matrices was noted in the other segments of the gastrointestinal tract.

3.3. Digestibility of nitrogen and amino acids

Nitrogen- and individual amino acid-digestibility values of the milk protein matrices are presented in **Table 3**. High values of nitrogen digestibility were found for the 4 milk protein matrices ranging from 93.5 ± 0.3% (for CW2) to 95.5 ± 0.3% (for C2 low Ca^{2+}) for orocaecal digestibility. Orocaecal nitrogen digestibility was significantly lower for the CW2 matrix in comparison to C1 ($P = 0.0405$), C2 low Ca^{2+} ($P = 0.0016$) and W ($P = 0.0094$). The same tendency was observed with the orofecal 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 was observed for the CW2 matrix, which had a significantly lower value compared to the other matrices ($P < 0.0001$). Indeed, the orocaecal digestibility was lower for the CW2 matrix for most of the individual amino acids except for methionine, alanine, glycine, and serine.

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 Ca^{2+}-, 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
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 Ca^{2+}- 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 Ca^{2+}-, 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 Ca^{2+}- 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.

3.5. Postprandial distribution of dietary nitrogen

The incorporation of dietary nitrogen into the body and urinary urea pools, organs and digestive tissues 6 h after meal ingestion, according to the different rat groups, is presented in **Table 4**. No significant difference was observed between the matrices regarding the quantity of dietary nitrogen transfer to body or urinary urea pool and consequently, no difference was found on the total deamination with values ranging from 7.8 ± 2.1% (for the C2 low Ca^{2+}) to 11.3 ± 4.8% (for the CW2 group). A significant effect of group was found on the incorporation of dietary nitrogen into 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 liver ($P < 0.0001$ for C1 and C2 low Ca^{2+}, and $P = 0.0066$ for CW2) and gastrocnemius muscle ($P = 0.0232$ for C1, $P = 0.0003$ for C2 low Ca^{2+} and $P = 0.0462$ for CW2) in comparison to the other groups. The incorporation of dietary nitrogen into the digestive mucosae was not different in the duodenum, but differences between groups were found in the more distal parts of the small 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 ingestion of the W matrix in comparison to the 3 others ($P < 0.0001$ for C1 and C2 low Ca^{2+}, and $P = 0.0372$ for CW2). In the ileum, incorporation of dietary nitrogen was significantly higher after ingestion of matrices containing whey (CW2 and W) in comparison to the C1 ($P = 0.0088$ and < 0.0001, respectively) and C2 low Ca^{2+} ($P = 0.0001$ and < 0.0001, respectively).

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 Ca^{2+} ($P < 0.0001$). 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 Ca^{2+}. The CW2 matrix also incorporated significantly more dietary nitrogen into plasma protein compared to the C2 low Ca^{2+} 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 Ca^{2+}, were observed.

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.

3.8. Postprandial plasma amino acid kinetics

We determined the kinetics of plasma amino acid concentrations from the fasting state to 6 h after ingestion of protein matrices (**Figure 6**). A significant effect of time was observed for the 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 meal ingestion and a return to basal level at 6 h. The casein-based matrices (C1 and C2 low Ca^{2+}) 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 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 concentration 1 h after meal intake in comparison to C1 (P = 0.0021) and C2 low Ca^{2+} (P = 0.0034).

4. Discussion

The aim of this study was to characterize the digestibility and postprandial utilization of ^{15}N-labeled 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 Ca^{2+}), a modified ratio of whey-to-micellar casein matrix (CW2) and a whey-based matrix (W).

Milk proteins are considered as high-quality proteins and are therefore known to display amongst 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 digestibility values from 92 to 97% in humans (Calvez et al., 2021; Gaudichon et al., 2002; Guillin 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., 2006; Rutherfurd, Fanning, Miller, & Moughan, 2015). Our results are partly in accordance with 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 caseins with low calcium (C2 low Ca^{2+}), respectively. We found comparable results for the native casein matrix in a previous study (Atallah et al., 2022). However, we observed a slightly lower 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; Rutherfurd et al., 2015). These slight discrepancies may be due to differences in methodology or in the whey protein used. We also studied a milk protein matrix that had a modified whey-to-micellar casein ratio (63: 37; CW2). Surprisingly, the rats consuming this matrix displayed a higher amount of dietary nitrogen in the caecum 6 h after ingestion, resulting in a slightly lower orocaecal digestibility for both nitrogen (93.5%) and mean amino acid (92.4%) in comparison to all the other matrices. The digestibility observed for this matrix was lower than that reported for total milk proteins in the literature. Indeed, total milk proteins which contain a casein-to-whey ratio of 80:20 had a true orocaecal nitrogen digestibility of 96% in rats (Lacroix, Leonil, et al., 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 accessibility to digestive enzymes in the CW2 matrix. Such an hypothesis is supported by the 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).

The duodenal, jejunal, ileal and caecal digesta were submitted to peptidomics analysis combining 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 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 MS/MS-scans recorded during the RP-HPLC MS/MS runs indicates that the number of ions, their intensity or both chosen for fragmentation in these samples are no more important in one run 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 from caecal content ranges from 50 ± 35 to 6 ± 5 according to the milk matrix ingested, and the number of identified peptides was significantly lower in the caecal digesta for the whey matrix compared to matrices containing mainly caseins. Recently, Sanchón *et al.* reported, the identification of 415 and 230 different peptides from human jejunal contents after casein and whey intake, respectively (Sanchon et al., 2018). Although human and rat models may not be directly comparable, the lower number of identified peptides from the rat caecum content correlates with the absorptive intestinal function of the small intestine. Moreover, the whey proteins, especially β-lactoglobulin, are reported to be resistant to pepsin action (Bouzerzour et 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 matrix ingested, suggesting the full gastrointestinal digestion and the full intestinal absorption of whey peptides. Inversely, although caseins are hydrolyzed within the first minutes of pepsin hydrolysis (Dupont & Tomé, 2020), some casein peptides are resistant to gastrointestinal digestion (Egger et al., 2017) and to epithelial proteases (Bauchart et al., 2007), and consistently, were found in the caecum content of 29 rats in our study. The gastrointestinal digestion resistance of such peptides is generally attributed to proline-containing peptides. In our study, among all the digestion-resistant peptides identified in the caecum, the occurrence of glutamate, valine, isoleucine, proline and serine were 330, 166, 118, 118 and 107, respectively. Numerous serine 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).

436 Finally, the protein backbone of αs1-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

mucosa of rats 6 h after meal intake. These different results could be explained by the difference in processing method used to obtain the milk protein powders, which could have in turn affected the protein structure (van Lieshout, Lambers, Bragt, & Hettinga, 2020). We also determined the 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. Values of postprandial deamination ranging from 8% to 20% have been previously reported depending of the size of the meal, the habitual protein intake and the time after meal ingestion 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 of dietary nitrogen to urea was quicker for whey proteins and reached a maximum 2 h after ingestion compared to caseins or total milk proteins, as reported in a human clinical trial (Lacroix, Bos, et al., 2006).

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 found in the gastrocnemius muscle and the liver compared to the other matrices. We found a FSR of proteins of around 100 - 110%/day in the liver and around 5 - 7%/day in the muscle 6 h after 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 was previously observed in energy-restricted rats that consumed meals containing either caseins 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 stimulation of muscle protein synthesis seemed to be highly dependent on the structure of the matrix and the composition in amino acids of the milk proteins (Reitelseder et al., 2011; Tang et 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 anabolism after different milk protein consumption in rats, the muscle protein FSR measured 4 h 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).

5. Conclusions

All the milk protein matrices tested in this study had high digestibility values. However, our results showed that milk proteins with a modified whey-to-micellar casein ratio displayed a slightly lower digestibility in comparison to caseins or whey proteins, probably due to an alteration in the 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 calcium content, showing that structural changes linked to demineralization of caseins did not 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 accordance with the rapid kinetics of whey proteins. Additionnally, no whey protein-derived peptides were identified in the rat caecal digesta. Indeed, the identified peptides, resistant to gastrointestinal digestion, belong only to αs1-, αs2- and β-caseins and are mainly located in the N-terminal half of the αs1-casein and the proximal N-term region of the β-casein. Hence, our 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 industrial processes on protein digestion and quality is thus critical to characterize the nutritional properties of innovative proteins.

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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.

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.

Data statement

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

Tables

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 Ca^{2+}	92	8	1.6	85
CW2	63	37	1.8	81
W	< 1	> 99	0.3	81

C: casein; W: whey. The matrices C1 and C2 low Ca^{2+} are casein-based but differ in their calcium concentration and the structure of casein micelles, while the CW2 matrix contains both casein and whey proteins in lower proportion as in cow milk. The matrix W is entirely composed of whey proteins. The total protein content was based on nitrogen content x 6.38.

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

* The protein source was the total milk proteins for the standard diet and correspond to the milk protein matrices in the experimental diets.

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 ± 0.3^{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 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 ± 0.3[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	*n.s.*
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	*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 [a]	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 ± sem, n = 8 rats/group. [1]Average 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.

Table 4. Transfer of dietary nitrogen to the urea pool and to the liver and gastrocnemius muscle (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 ± 0.7[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	*n.s.*
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*

Values are mean ± sem, n = 8 rats /group. [1] expressed as % of ingested nitrogen /organ. [2] expressed as % of dietary nitrogen incorporated in 100 mg of mucosae. 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.

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 ± 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 Ca^{2+}-, 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 Kruskall-
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 αS1-
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 αS1-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 ± sem, n = 8 rats. The group- and protein matrix-effects were tested through

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

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

Figure 6. Plasma concentrations of total amino acids (A), indispensable amino acids (B), and leucine (C). Values are expressed as mean ± 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

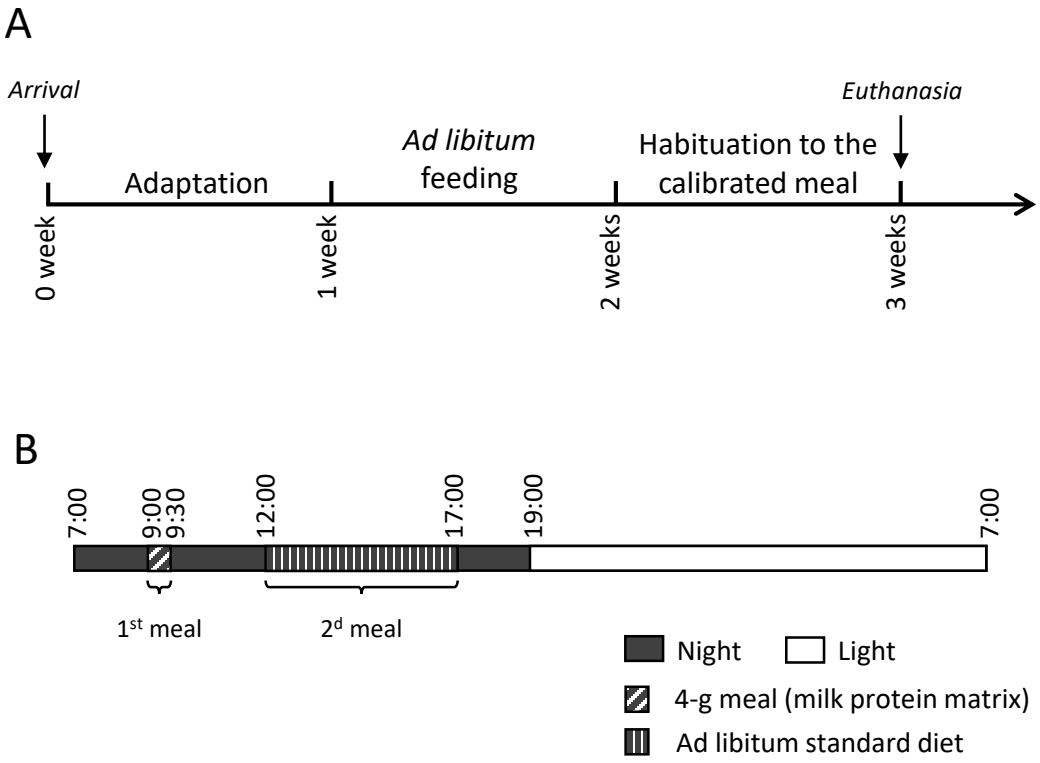

A
Arrival
Euthanasia
Adaptation
Ad libitum feeding
Habituation to the calibrated meal
0 week
1 week
2 weeks
3 weeks
B
7:00
9:00
9:30
12:00
17:00
19:00
7:00
1st meal
2d meal
Night
Light
4-g meal (milk protein matrix)
Ad libitum standard diet

Figure 2

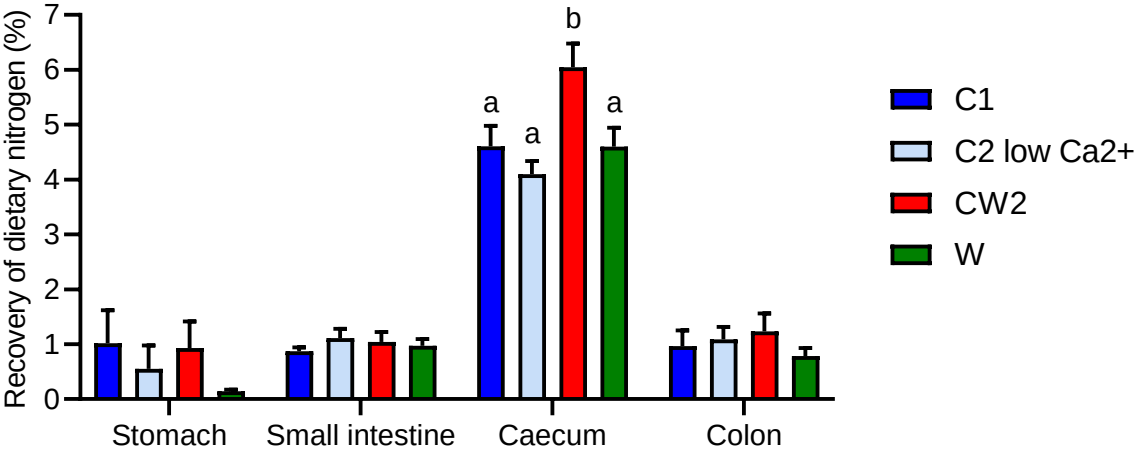

Recovery of dietary nitrogen (%)
0
1
2
3
4
5
6
7
a
a
b
a
Stomach
Small intestine
Caecum
Colon
C1
C2 low Ca2+
CW2
W

Figure 3

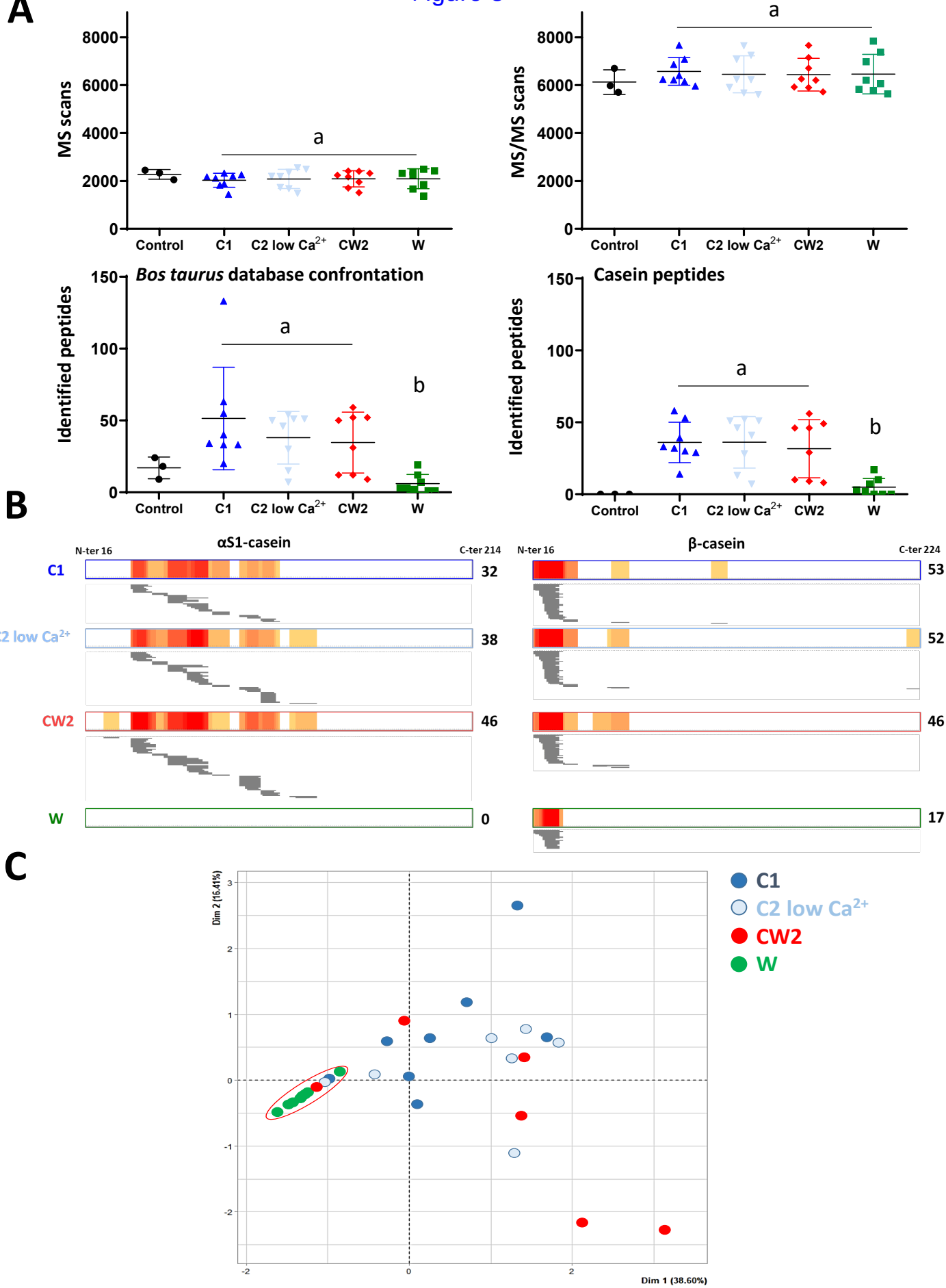
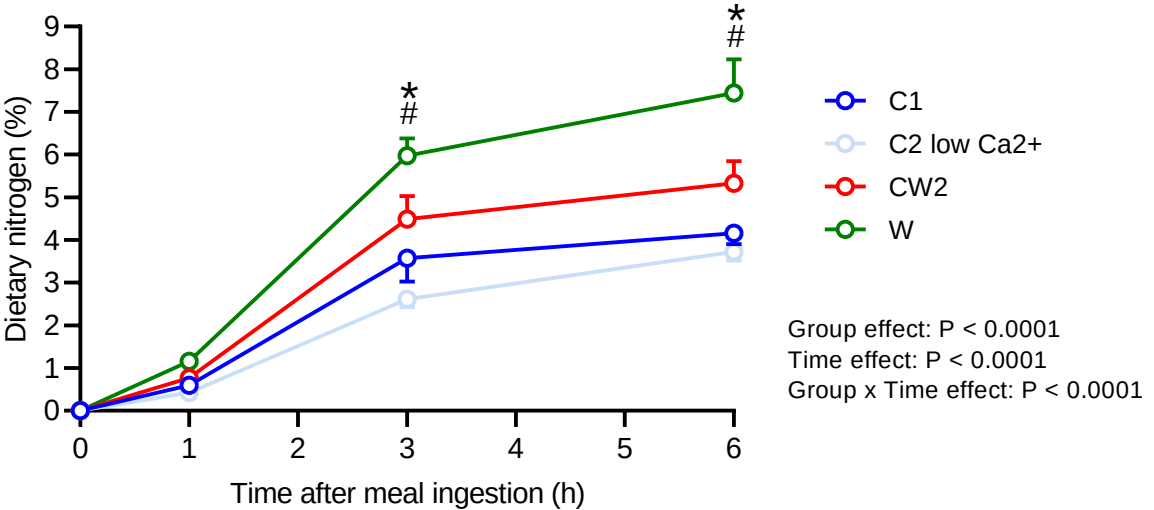

A
MS scans
8000
6000
4000
2000
0
a
Control
C1
C2 low Ca2+
CW2
W
MS/MS scans
a
Identified peptides
Bos taurus database confrontation
150
100
50
0
a
b
Casein peptides
B
αS1-casein
N-ter 16
C-ter 214
C1
32
C2 low Ca2+
38
CW2
46
W
0
β-casein
N-ter 16
C-ter 224
53
52
46
17
C
Dim 2 (16.41%)
Dim 1 (38.60%)
C1
C2 low Ca2+
CW2
W

Figure 4



Group effect: P < 0.0001
Time effect: P < 0.0001
Group x Time effect: P < 0.0001

Figure 5

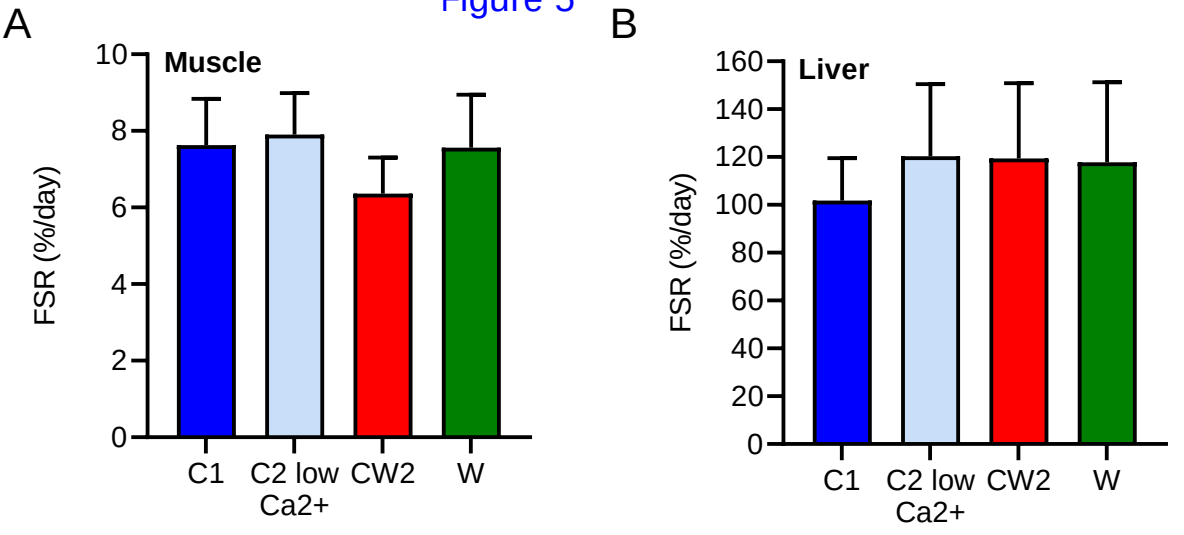
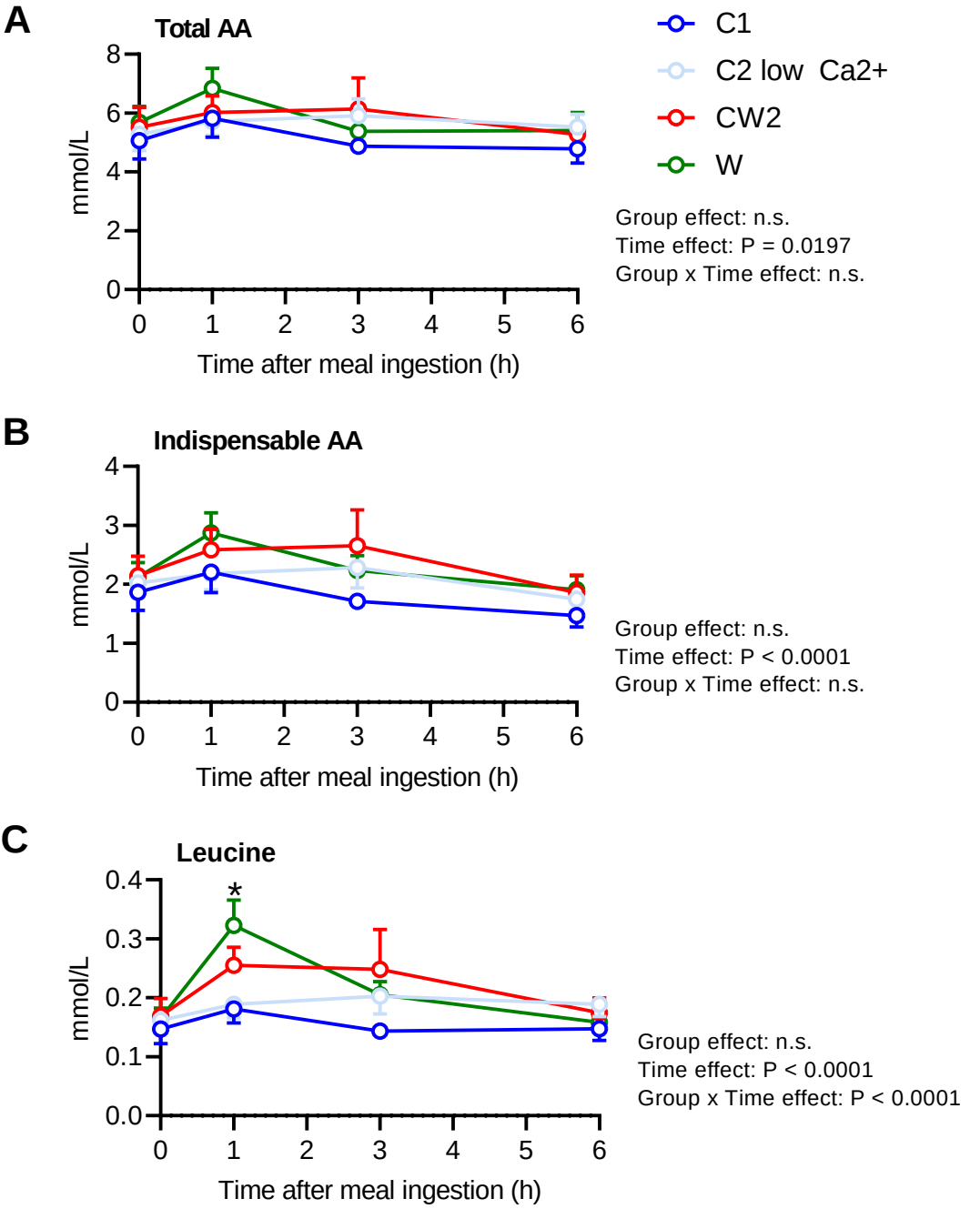

A
Muscle
FSR (%/day)
10
8
6
4
2
0
C1
C2 low Ca2+
CW2
W
B
Liver
FSR (%/day)
160
140
120
100
80
60
40
20
0
C1
C2 low Ca2+
CW2
W

Figure 6


A
Total AA
mmol/L
Time after meal ingestion (h)
C1
C2 low Ca2+
CW2
W
Group effect: n.s.
Time effect: P = 0.0197
Group x Time effect: n.s.
B
Indispensable AA
mmol/L
Time after meal ingestion (h)
Group effect: n.s.
Time effect: P < 0.0001
Group x Time effect: n.s.
C
Leucine
mmol/L
Time after meal ingestion (h)
Group effect: n.s.
Time effect: P < 0.0001
Group x Time effect: P < 0.0001

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

Nathalie Atallah[123], Claire Gaudichon[1], Audrey Boulier[2], Alain Baniel[2], Benoit Cudennec[3], Barbara Deracinois[3], Rozenn Ravallec[3], Christophe Flahaut[3], Dalila Azzout-Marniche[1], Nadezda Khodorova[1], Martin Chapelais[1], Juliane Calvez[1]

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

[2]Ingredia S.A. 62033 Arras Cedex, France

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

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

	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

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