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Influence of food structure on dairy protein, lipid and calcium bioavailability: A narrative review of evidence

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2 1 **Influence of food structure on dairy protein, lipid and calcium bioavailability: A**
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4 2 **narrative review of evidence**

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22 **Short title:** *Dairy food structure and nutrient bioavailability*

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Abstract

Beyond nutrient composition matrix plays an important role on food health potential, notably acting on the kinetics of nutrient release, and finally on their bioavailability. This is particularly true for dairy products that present both solid (cheeses), semi-solid (yogurts) and liquid (milks) matrices. The main objective of this narrative review has been to synthesize available data in relation with the impact of physical structure of main dairy matrices on nutrient bio-accessibility, bioavailability and metabolic effects, *in vitro*, in animals and in humans. Focus has been made on dairy nutrients the most studied, *i.e.*, proteins, lipids and calcium. Data collected show different kinetics of bioavailability of amino acids, fatty acids and calcium according to the physicochemical parameters of these matrices, including compactness, hardness, elasticity, protein/lipid ratio, P/Ca ratio, effect of ferments, size of fat globules, and possibly other qualitative parameters yet to be discovered. This could be of great interest for the development of innovative dairy products for older populations, sometimes in protein denutrition or with poor dentition, involving the development of dairy matrices with optimized metabolic effects by playing on gastric retention time and thus on the kinetics of release of the amino acids within bloodstream.

Keywords: Dairy products, food structure, protein, lipid, calcium, bio-accessibility, bioavailability

53 Introduction

54 Today there are sufficient scientific evidences to include the matrix effect for defining food health
55 potential, not only nutrient composition (Fardet, 2014a; Fardet, 2015a; Fardet, 2015b; Fardet et al.,
56 2013). Indeed, at similar composition (nutrients and calories) two foods with distinct matrix
57 structure - resulting from either different technological processes or different degree of chewing -
58 may give different health potential, as shown for example with apple products (Haber et al., 1977),
59 durum wheat pasta versus bread (Granfeldt et al., 1991), whole carrot versus carrot nutrients
60 (Moorhead et al., 2006), almonds of different particle sizes (Grundy et al., 2016), casein versus
61 corresponding aminoacid mixture (Dangin et al., 2001) and grilled beef meat differently chewed
62 (Rémond et al., 2007). Concerning the carbohydrate fraction, according to starch structure (Fardet,
63 2015b), food particle size (Holt and Miller, 1994) and/or food density (Burton and Lightowler,
64 2006) it is well recognized that blood glycaemic responses may be rather different leading to
65 different insulinemic responses with important consequences for diabetic subjects (Fardet, 2014b).
66 Concerning the protein fraction, it has also been shown that depending on chewing (Rémond et al.,
67 2007) or protein structure (Boirie et al., 1997) the subsequent protein metabolism and protein gain
68 are different, also with important implications, notably in elderly populations (Dangin et al., 2003).
69 Finally, the same is true for the lipid fraction, and depending on lipid droplet size, lipids are not
70 metabolized similarly with again implication in clinically ill patients, as for lipase pancreatic
71 insufficiency (Armand et al., 1999). On the other hand, concerning the fiber fraction, depending on
72 their physico-chemical properties such as water-holding capacity, porosity, swelling behaviour
73 and/or degree of crystallinity, they are not fermented according to the same profiles leading to
74 production of different short-chain acid profiles within colon (Fardet, 2016). Therefore, for all these
75 important nutrient it is not exaggerated to define slow and rapid fractions, each one impacting
76 differently metabolism. In all these examples this is not the composition which matters but the
77 physicochemical characteristics of the food matrix. If such effects have been widely studied for
78 plant-based foods this is less true for animal-based foods, notably dairy products. Yet, main dairy
79 products exhibit three different types of food structure or texture as defined by rheological science,
80 i.e., liquid (milks and some fermented milks), semi-solid (yogurts and some fresh cheeses) and solid
81 (most of cheeses). And in each category are encountered numerous different food structures,
82 especially in cheeses.

83 Otherwise it is not only to know the food composition to estimate which nutrient fraction
84 will be actually used by the organism. For example, in starchy foods, a fraction of starch is not
85 digested within small intestine and arrived to the colon where it is fermented as resistant starch
86 fraction leading to the production of butyric acid (Birt et al., 2013). In brief, foods are first broken
87 down during chewing and they release within mouth, stomach and small intestine a fraction of their

1 88 nutrient which is called the bioaccessible fraction (Fardet, 2015b). Secondly, this fraction is not
2 89 always fully absorbed and reaches the colon. Thirdly, the absorbed fraction is not always fully
3 90 metabolized and used by organism, a fraction being released in urine *via* the kidney, *e.g.*, as for
4 91 polyphenols (Scalbert et al., 2002). The final fraction really used by our organism and exerting a
5 92 defined physiological function is what is generally called the bioavailable fraction.

6 93 The bioaccessible fraction is generally measured *via* dynamic or static *in vitro* digestive
7 94 systems more or less mimicking that of humans (McClements and Li, 2010). In some cases a
8 95 semipermeable (cellulose) membrane or dialysis bag is added allowing to measure a dialysable
9 96 fraction or *in vitro* bioavailable fraction (Bouayed et al., 2012). Sometimes this is coupled with
10 97 model cells such as Caco2 cells allowing to estimate the fraction uptake by the cell (Haraldsson et
11 98 al., 2005). *In vivo*, comparing what is ingested with what is excreted or collected in feces allows
12 99 estimating an apparent digestibility (Kristensen et al., 2008), generally performed in animals (Li et
13 100 al., 2016). But to reach the real bioavailable fraction in humans, more expensive studies are needed,
14 101 measuring nutrient content in plasma (Fenech et al., 1999), golden standard studies involving
15 102 radiolabelled foods with isotopes to distinguish between exogenous and indigenous nutrient (Bruno
16 103 et al., 2006). And these latter studies are very rare. However, in these studies the initial food is
17 104 generally consumed alone whereas other foods may interact and modify the bioavailable fraction.

18 105 The impact of food structure on health is therefore an important research topic, but still in
19 106 progress. Notably there is no review about the impact of dairy food structure on their health
20 107 potential. Therefore the main objective of this narrative review has been to synthesize available data
21 108 in relation with the impact of physical structure of main dairy matrices (*i.e.*, milks, yogurts,
22 109 fermented milks and cheeses) on nutrient bio-accessibility, bioavailability and metabolic and health
23 110 effects, *in vitro*, in animals and in humans. A particular focus has been made on dairy nutrients the
24 111 most studied, *i.e.*, proteins, lipids and calcium.

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114 **Dairy food structure and macronutrient bioavailability**

115 ***Food matrix disintegration and the protein fraction***

116 *In vitro* studies

117 *Milks, yogurts and fermented milks*

118 Seven studies investigating the digestive fate of milks (Almaas et al., 2006; Dupont et al., 2010;
119 Rinaldi et al., 2014) (Kopf-Bolanz et al., 2012; Tunick et al., 2016; Ye et al., 2016), fermented milk
120 (Matar et al., 1996) and yogurts (Dupont et al., 2010; Rinaldi et al., 2014) have been identified.
121 Concerning milks, different types of milks were compared : goat *versus* cow milks (Almaas et al.,
122 2006), pasteurized and homogenized milk (Kopf-Bolanz et al., 2012), heated *versus* raw milk

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2 123 (Almaas et al., 2006; Ye et al., 2016), and pasteurized *versus* sterilized milks (Almaas et al., 2006;
3 124 Dupont et al., 2010; Rinaldi et al., 2014).

5 125 Before comparing different milks it is worth beginning with the study of the mere *in vitro*
6 126 gastrointestinal digestion of a whole pasteurized and homogenized milk (Kopf-Bolanz et al., 2012).
7 127 After oral and gastric digestion, all proteins except β -lactoglobulin were degraded, with peptides
8 128 being further degraded at the end of intestinal digestion. Most identified peptides came from
9 129 digestive enzymes and only a few from milk. Concerning the β -lactoglobulin authors suggested that
10 130 fatty acids and triglycerides would link to it preventing proteases access, and therefore requesting
11 131 bile for further digestion (Gass et al., 2007). Indeed, when *in vitro* digestion was completed 54% of
12 132 total proteins were degraded into free amino acids, dipeptides and tripeptides, and only 43% without
13 133 bile. Average peptide sizes was of 5-6 aminoacids. Finally while undigested milk contains 45%
14 134 essential aminoacids, the free aminoacids coming from the 10% protein fraction degraded after
15 135 complete digestion contains 94% essential aminoacids.

16 136 Compared to skimmed cow milk, skimmed caprine goat milk proteins are digested faster,
17 137 caprine β -lactoglobulin being less resistant to digestion (23% remaining after gastro-duodenal
18 138 digestion) than cow milk (83% remaining) (Almaas et al., 2006). Difference in tertiary structure and
19 139 surface hydrophobicity could be involved in such differences (El-Zahar et al., 2005). For goat milk,
20 140 milks with high and low content (typical of some Norwegian goats) in α_{S1} -casein were also studied,
21 141 but no significant difference was observed for protein digestion. Upon heating (pasteurization at
22 142 72°C for 15 seconds or sterilization at 100°C for one minute) protein digestion was lower with both
23 143 caprine and ovine milks due to protein denaturation and aggregation through heating. However,
24 144 while a higher degradation of the pasteurized milk than the high heated milk proteins was observed
25 145 for cow milk there was no difference for goat milk.

26 146 Heating milk has important impact on the hydrolysis kinetics of its protein fraction. In a
27 147 recent study Ye and Sui have investigated the *in vitro* gastric digestion of unheated and heated
28 148 (90°C for 20 minutes) skimmed milk (Ye et al., 2016). Both milks formed a gastric clot after 10
29 149 minutes digestion but with very different structures: while unheated milk « showed a closely knitted
30 150 network with numerous small pores interspersed throughout the matrix » heated milk exhibited « a
31 151 network structure with larger voids ». Contrary to unheated milk the heated milk clot includes both
32 152 casein and whey proteins within a loose structure. As a result, casein digestion is much slower, and
33 153 β -lactoglobulin and α -lactalbumine remained globally intact with unheated milk. In heated milk
34 154 whey proteins were readily hydrolyzed and only peptides were released within small intestine.
35 155 Upon longer time of gastric digestion clot structures tightened and became less permeable
36 156 consequently influencing casein digestion by pepsin. Clot formation is therefore initially governed
37 157 by the action of pepsin towards casein κ leading to the destruction of the protective effect that

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2 158 casein κ have on casein micelles. Heating clearly influences clot structure and this may find
3 159 application through the creation of clot with different structures to control protein bioavailability
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5 160 and also of non protein compounds to influence glyceimic, lipidic and aminoacid metabolisms.

6 161 Rinaldi *et al.* went further by studying *in vitro* gastrointestinal protein digestibility of three
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8 162 commercial distinct dairy matrices, *i.e.*, sterilized and pasteurized milks (liquid matrices), and
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10 163 stirred-yogurt (semi-solid matrix) (Rinaldi et al., 2014). As suggested in the previous study, the
11 164 differences in both caseins and whey protein digestions were mainly driven by the gastric digestion
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13 165 phase. After buccal digestion, soluble proteins were lower with yogurt than with milks but the
14 166 difference decreased at the end of gastric digestion and disappeared after duodenal digestion.
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16 167 However, between each phase of digestion, the highest increase in soluble proteins was observed
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18 168 with yogurt. Casein digestion began in stomach and was slower for pasteurized milk than with
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20 169 sterilized milk and yogurt. At the end no casein was present after duodenal digestion and traces of
21 170 whey proteins were observed for pasteurized milk and yogurt. Besides, the release of free
22 171 aminoacids during the duodenal phase varied according to their nature (acid, basic, neutral or
23 172 hydrophobic) and seemed to be driven by enzyme specificity, but hydrophobic aminoacids were
24 173 released at higher amounts for all matrices. On the other hand, a greater proportion of free
25 174 aminoacids and short peptides was released from sterilized milk than from other dairy matrices;
26 175 which leads authors to suggest that more than food structure, processing, especially heating, had
27 176 impact on protein digestion. Hence the importance of a good mastery of heating process.

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29 177 The degree of complexity was still increased in the following study in which a yogurt, three
30 178 raw (whole, homogenized, skimmed), two pasteurized (82°C for 30 seconds, whole, homogenized)
31 179 and three sterilized (120°C for 10 minutes, whole, homogenized, semi-skimmed) milks were
32 180 manufactured from the same original milk batch, and subjected to *in vitro* digestion using an infant
33 181 gut model (Dupont et al., 2010). The primary objective of this study was to determine whether
34 182 processing could modify the resistance of caseins to digestion and to identify resistant regions
35 183 capable of eliciting an allergic response in infant, not to study the protein digestive fate of each
36 184 dairy product. Globally caseins resisted digestion, especially casein κ and α_{s1} due to hydrophobic
37 185 fragments and/or post-translational modifications such as phosphorylation and glycosylation. Thus,
38 186 at gastric level it seems that there was a relationship between hydrophobicity at pH 3.0 (gastric pH)
39 187 and resistance to digestion, each type of casein having its own hydrophobic fraction: 149-199 for
40 188 casein α_{s1} , N terminal moiety for casein α_{s2} , 55-92 for casein β and 120-169 for casein κ ; these area
41 189 becoming more hydrophilic at duodenal level and therefore more accessible to digestive enzyme.
42 190 Otherwise, milk processing leads to different peptide profiles and milk heating increased the
43 191 number of peptides in digested samples (as shown in previous study). For all three matrices, at
44 192 gastric level intact casein all disappeared after 20 minutes of digestion, and β -lactoglobulin was

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2 193 more digested with yogurt. Authors identified 19 peptides from casein α_1 with raw milk, 16 for
3 194 pasteurized milk and 51 for yogurt. Finally it was concluded that milk processing increases casein
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5 195 resistance to digestion in the condition of this study.

6 196 Subsequently, the study of Tunick *et al.* (Tunick et al., 2016) investigated the role of milk
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8 197 processing treatments on proteins and lipids digestion. Raw skim milk, raw whole milk,
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10 198 homogenized raw whole milk, pasteurized whole milk, homogenized HTST-pasteurized whole
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12 199 milk, homogenized UHT-sterilized whole milk, HTST-pasteurized skim milk and UHT-sterilized
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14 200 skim milk (Tunick et al., 2016) were prepared. Before digestion, the protein profiles of most milks
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16 201 were similar except for homogenized UHT-sterilized whole milk and UHT-sterilized skim milk
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18 202 which showed a smearing of the bands indicative of new disulfide bond formation (Wada and
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20 203 Loennerdal, 2014). During gastric digestion, homogenized UHT-sterilized whole milk and UHT-
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22 204 sterilized skim milk showed faded bands for β -lactoglobulin and darker bands for the peptides
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24 205 below 5 kDa. This is in agreement with previous study (Rinaldi et al., 2014) where denatured β -
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26 206 lactoglobulin is sensitive to pepsin hydrolysis favoring the production of smaller peptides. Also,
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28 207 whole milks and skim milks exhibited different protein digestion profiles. Independently of the
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30 208 processing treatment applied, fader protein bands were observed for skim milk samples during
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32 209 gastric and duodenal digestion suggesting a faster protein bioaccessibility. For skim milk, HTST-
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34 210 pasteurized or UHT-sterilized have no effect on protein digestion according to the SDS-PAGE
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36 211 protein profile. However for whole milks, the processing treatments have an impact. Homogenized
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38 212 HTST-pasteurized whole milk was more resistant during intestinal digestion compared to the others
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40 213 milks. This may be explained by the residual large fat droplets stabilized by proteins found at the
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42 214 end of the intestinal digestion for homogenized HTST-pasteurized whole milk. This could not be
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44 215 confirmed for homogenized UHT-sterilized whole milk as the confocal microscopy images were
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46 216 not presented by the authors.

47 217 It is obvious that thermal processing have important impact on the kinetics of nutrients
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49 218 release (peptide production). But altering the composition by modifying the casein to whey protein
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51 219 ratio (Rioux and Turgeon, 2012) or by the addition of stabilizer (Rinaldi et al., 2015) may also
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53 220 affect protein digestion. Indeed, three yogurt formulations with casein to whey protein ratios of
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55 221 4.5:1 (ratio of regular milk), 2.8:1 (ratio usually used in the yogurt industry) and 1.5:1 (ratio
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57 222 containing more whey proteins) were formulated (Rioux and Turgeon, 2012). Yogurt with a 1.5:1
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59 223 ratio was more hardly digested. The heat treatments used during yogurt-milk processing favors the
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61 224 formation of denatured whey proteins which have the capacity to form large aggregates or
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63 225 complexes with κ -casein. These were proposed to have a larger size in yogurts with a 1.5:1 ratio,
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65 226 affecting pepsin proteolytic activity on caseins. The addition of stabilizers (starch, pectin or β -
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67 227 glucan) also modifies milk protein digestion during the gastric phase (Rinaldi et al., 2015). Faster

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2 228 proteolysis, lower release of large peptides, and a higher proportion of free amino acids was found
3 229 for yogurts formulated with pectin or β -glucan than those with starch or without stabilizer.
4 230 Stabilizers and proteins are not always compatible when found in mixture and sometimes they
5 231 segregate (found in separate phases). It was hypothesized that β -glucan and dairy proteins segregate
6 232 during *in vitro* gastric digestion explaining the faster proteolysis found due to concentration of
7 233 pepsin and proteins in a separated phase. This could be considered as “micro-reactors” favoring
8 234 protein digestion. In milk beverage enriched or not with alginate (negatively charged
9 235 polysaccharides) or konjac glucomannan (neutral polysaccharides), milk proteins digestion was
10 236 reduced (Borreani et al., 2016). Under the condition studied, alginate is interacting with dairy
11 237 proteins preventing pepsin hydrolysis explaining a decrease in protein digestion compared with the
12 238 other beverages.

13 239 In the last study Matar *et al.* evaluated the effect of lactic acid bacteria on protein hydrolysis
14 240 during milk fermentation (by *Lactobacillus helveticus*) and on the subsequent release of
15 241 aminoacids and peptides after simulated peptic and pancreatic digestion (Matar et al., 1996). Fresh,
16 242 unpasteurized skim milk, pasteurized skim milk, pasteurized skim milk containing cells of
17 243 *Lactobacillus helveticus*, pasteurized skim milk fermented for 12 h with *Lactobacillus helveticus*
18 244 with pH controlled at 6, and pasteurized skim milk fermented for 12 h with *Lactobacillus helveticus*
19 245 without pH control were tested upon *in vitro* digestion. Overall, there were already free amino acids
20 246 in fermented milk, and milk fermentation impacted the release of some amino acids during *in vitro*
21 247 digestion, *e.g.*, the availability of proline. In milk, proline release is very slow due to the resistance
22 248 of imine bond (*i.e.*, compound containing a carbon-nitrogen double bond) to pepsin and pancreatin
23 249 activities. Specific proteolytic activity of *Lactobacillus helveticus* leads to faster release of proline
24 250 and short peptides containing proline. Fermentation also favours modification of protein structure
25 251 and the release of peptides having different hydrophobicity. The bacterial activity also generated
26 252 some bioactive peptides such as peptide 114-119 from casein β and peptide 98-106 from casein κ .
27 253 Authors concluded that the fermentation of milk by proteolytic bacteria may therefore provide a
28 254 new mechanism leading to the release of novel peptides from milk proteins after digestion.

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30 256 *Cheeses*

31 257 Cheese matrices are complex in both structure and composition. Recently Fang *et al.* have carried
32 258 out two *in vitro* digestion studies on different cheeses varying in texture and fat content (Fang et al.,
33 259 2016a; Fang et al., 2016b).

34 260 The first study have been led with five commercial cheeses, covering a range of textural
35 261 properties: a Camembert (soft texture, but described as "firm" in this study), a smear cheese, a
36 262 young Cheddar (uncooked pressed cheese), an aged Cheddar and a string Mozzarella (Fang et al.,

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2 263 2016a). After gastric digestion disintegration and proteolysis profiles were different according to
3 264 cheeses considered. Disintegration was higher when initial hardness, cohesiveness and chewiness
4 265 were lower. Thus the Camembert needed more strength for its matrix to be broken up. The buccal
5 266 disintegration was already rapid then gastric pepsinolysis decreased cohesive strengths that
6 267 maintain the matrix, increasing furthermore disintegration. For mozzarella, initial cohesiveness and
7 268 hardness were higher leading to a slower and continuous gastric disintegration. Otherwise, an
8 269 important result was that fat content and proteolysis impacted textural characteristics that in return
9 270 impacted disintegration; and cheese disintegration content was positively correlated with fat
10 271 content. Thus elastic mozzarella was three times less disintegrated than matured soft cheese
11 272 (Camembert and aged Cheddar) with higher fat contents. Then, peptides/protein releases were in
12 273 agreement with disintegration kinetics, *i.e.*, two-fold less with mozzarella. Overall at the end of
13 274 orogastric digestion Camembert and mozzarella were disintegrated by around 75 and 18%,
14 275 respectively while peptide/protein releases were of around 40 and 17%, respectively. Duodenal
15 276 digestion exhibited a very important role on disintegration and proteolysis so that there were no
16 277 more significant difference for disintegration and peptide/protein release after both 5 and 180
17 278 minutes digestion for Camembert and mozzarella. The results of this first study well showed that it
18 279 is possible to control cheese manufacturing parameters, *i.e.*, structure and texture, to modulate
19 280 further protein digestion and impact post-prandial proteic metabolic response.

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31 281 In the second study, the effect of fat content and texture was further investigated through *in*
32 282 *vitro* digestion of Cheddar (33% fat), light Cheddar (21% fat), Mozzarella (20% fat) and light
33 283 Mozzarella (14% fat) cheese particles (Fang et al., 2016b). For the four cheeses the disintegration
34 284 profiles were clearly modulated by texture. Thus at the end of gastric digestion, considering all
35 285 cheeses together disintegration was negatively correlated with hardness, cohesiveness, resilience,
36 286 fat content, proteolysis degree, chewiness and adhesiveness. At the end of duodenal digestion,
37 287 disintegration was negatively correlated with hardness, elasticity, cohesiveness, resilience,
38 288 proteolysis degree and chewiness. Again firmer and more cohesive cheeses were disintegrated more
39 289 slowly, and nitrogen compounds followed disintegration profiles. Disintegration continue at
40 290 duodenal level but the time required for complete disintegration differed according to cheeses.
41 291 Without enzymes, disintegration occurred but it was weaker with an average of around 75% with
42 292 enzymes and of around 30% without enzymes. Concerning the protein release there was no
43 293 significant difference after oral digestion; while after gastric digestion it was the highest with
44 294 regular Cheddar (\approx 52%) and the lowest with light Mozzarella (\approx 40%). Differences were leveled
45 295 after duodenal digestion but regular Cheddar always exhibited a significantly higher level of
46 296 disintegration than three other cheeses. Finally, duodenal cheese disintegration was negatively
47 297 predicted (total variance explained: 93%) by solid non-fat, chewiness, hardness and resilience.

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2 298 Authors reached the same conclusion than in their first study, *i.e.*, « the modification of fat content
3 299 in cheese, affecting cheese texture and microstructure, could regulate the protein digestion, which
4 300 may further affect acute protein metabolic response and some physiological functions » (Fang et al.,
5 301 2016b).

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8 302 Similar results were obtained by the same research team in 2012 (Lamothe et al., 2012).
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10 303 Mild Cheddar (31% lipid), aged Cheddar (34% lipid), light Cheddar (23% lipid) and Mozzarella
11 304 (25% lipid) cheeses were also submitted to *in vitro* gastro-intestinal digestion and investigated for
12 305 the association between texture and disintegration rate and extent. After two hours of gastric
13 306 digestion aged Cheddar was clearly the more rapidly disintegrated compared to other three cheeses
14 307 (≈ 65 versus $\approx 20-30\%$); but differences were again leveled after two hours duodenal digestion even
15 308 if kinetics differed. In all cheeses tested, the most important changes in matrix disintegration were
16 309 observed within the first 30 min of the duodenal phase. Finally the fat content also affected
17 310 degradation in the duodenal phase.

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22 311 Qureshi *et al.* studied *in vitro* gastrointestinal protein digestibility of two other types of
23 312 cheese (Norwegian): the Gamalost (granular cheese, ripened for 10 and 30 days) and the Norvegia
24 313 (hard cheese, ripened for 90 days) (Qureshi et al., 2013). The Gamalost is prepared by boiling milk
25 314 of lean cow without adding rennet, contrary to what is customary in Europe. The Norvegia cheese is
26 315 a trademark of cheese with secret recipe and close to Gouda. It melts easily with heat. The two
27 316 cheeses therefore have different matrices. Overall Gamalost cheese gave more peptides after
28 317 digestion than for Norvegia cheese, most of them derived from β -casein, some of casein α_{s1} and
29 318 only a few from casein α_{s2} and κ . After digestion, both cheeses led to increase amounts of released
30 319 aromatic and positively charged amino acids, and leucine; but considering all amino acids,
31 320 Gamalost cheese released higher quantities than Norvegia cheese. Some peptides remain intact after
32 321 digestion and more peptides were generated after gastric than duodenal digestion. Such results again
33 322 emphasized an important matrix effect for cheeses with different initial texture.

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36 323 In the end, cheeses were more resistant to protein and lipid digestion than liquid or semi-
37 324 solid matrices were. No direct relationship could be established between disintegration kinetics and
38 325 cheese rheological properties (Lamothe et al., 2017).

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42 327 Animal studies

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44 328 The matrix effect was first tested in minipigs consuming yogurt or milk (Gaudichon et al.,
45 329 1994). This study was of great interest because it was a *sensu stricto* bioavailability study using a
46 330 radiolabeled yogurt and milk with nitrogen ^{15}N allowing differentiation between endogenous and
47 331 exogenous nitrogen. First, there was no effect of both dairy matrices on nitrogen endogenous
48 332 secretions. Compared to milk, yogurt delayed intestinal release of liquid phase and of chyme

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2 333 nitrogen fraction. One hour after consumption, 84 and 64% of exogenous nitrogen remained in the
3 334 stomach for yogurt and milk, respectively; but after four hours more yogurt nitrogen remained in
4 335 the stomach (19%) compared to milk (32%). Overall both matrices were highly digestible as 93% of
5 336 exogenous nitrogen has disappeared twelve hours after meal. The most striking result was the high
6 337 correlation between absorbed exogenous nitrogen and kinetics of released nitrogen at intestinal
7 338 level ($r = 0.999$ for milk and $r = 0.974$ for yogurt). Once in intestine milk proteins were rapidly
8 339 absorbed. Gastric emptying therefore appears to be a major factor for controlling the kinetics of
9 340 nitrogen absorption, the higher viscosity of yogurt allowing a more regular release of nitrogen into
10 341 intestine leading to a more gradual absorption and distribution of dietary nitrogenous compounds
11 342 within organism.

12 343 The next study, also carried out in minipigs, is also particularly relevant for evaluating the
13 344 impact of dairy food matrices on protein digestion because from the same ULH (“ultra low heat”)
14 345 skimmed milk powder were processed four different matrices: 1) heated liquid skimmed milk (90°C
15 346 for 10 minutes); 2) unheated liquid skimmed milk; and 3-4) corresponding dairy gels (coagulation
16 347 with rennet at 20°C for 24 hours) (Barbé et al., 2013). First dairy gels slowed down the flow of the
17 348 meal from stomach with low level of proteins at duodenal level. This consequently slowed down
18 349 amino acid absorption and bioavailability. In the stomach casein and β -lactoglobulin from unheated
19 350 dairy matrices were sensitive and resistant, respectively, to hydrolysis; whereas with heated dairy
20 351 matrices they all exhibited similar digestion β -lactoglobulin becoming susceptible to hydrolysis.
21 352 Compared to other matrices, casein from heated gel seemed slightly more retained in the stomach.
22 353 With heated liquid matrix β -lactoglobulin showed a fast duodenal appearance while being very low
23 354 for gels. Otherwise liquid versus solid matrices had not the same effect on CCK (anorexigenic) and
24 355 ghrelin (orexigenic): for example the increase in CCK (cholecystochinine) concentration was faster
25 356 with unheated liquid dairy matrix than with unheated gel; but unheated gel induced a more
26 357 prolonged increase in CCK than unheated liquid matrix. Finally post-prandial decrease in ghrelin
27 358 concentration was more pronounced with unheated gel than with unheated liquid. The main
28 359 conclusion of this study was that both microstructure and macrostructure were affected by heating
29 360 and gelification processes, respectively, and that both impacted differently proteolysis steps,
30 361 microstructure acting more on hydrolysis and macrostructure more on amino acid absorption and
31 362 bioavailability.

32 363 The following year Barbé *et al.* published another study in multi-cannulated minipigs to
33 364 investigate the protein digestion of two distinct dairy gel matrices with the same composition,
34 365 similar rheological and structural properties but differing for their type of coagulation (acidification
35 366 *versus* renneting) (Barbé et al., 2014b). While the acid gel led to a duodenal peak of casein and β -
36 367 lactoglobulin after only 20 minutes of digestion and to a peak of plasmatic amino acids after 60

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2 368 minutes of digestion the rennet gel led to lower levels of duodenal proteins and plasmatic amino
3 369 acids. In addition the rennet gel appeared more satietogenic (plasma CCK). Authors proposed the
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5 370 following explanation for the observed effects, *i.e.*, a combined action of three mechanisms: 1) a
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7 371 strong resistance of the rennet gel to hydrolysis in the stomach, 2) combined with a longer gastric
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9 372 retention time, and 3) a substantial dilution by gastric juices; besides, while acid gel flocculated in
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11 373 stomach without apparent syneresis the rennet gel exhibited an extensive syneresis indicating a
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13 374 strong contraction of gel particles leading to a more pronounced retention and a delayed gastric
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15 375 emptying. In addition particle contraction has limited enzyme accessibility to cleavage sites of
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17 376 proteins (Kong and Singh, 2008). Therefore, the time of gastric residence might induce satiety with
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19 377 potential application in developing diets for loosing weight in case of obesity (Kong and Singh,
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21 378 2008). On the contrary, the impacted bioavailability of amino acids may have a potential effect on
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23 379 splanchnic and peripheric protein metabolism with potential application in elderly populations for
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25 380 which the preference for acidified milk would induce a more efficient muscular synthesis.

26
27 381 In their third study Barbé *et al.* went further through identification (by LC-MS/MS) of
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29 382 released peptides in the gastrointestinal tract of minipigs from six distinct dairy matrices with
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31 383 similar composition but with different physical structure (Barbé *et al.*, 2014a). Duodenal effluents
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33 384 were collected upon five hours after consumption of the six following dairy matrices (which were
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35 385 prepared from Ultra Low Heat (ULH) skim milk powder): 1) heated milk (90°C for 10 minutes), 2)
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37 386 raw milk, 3-4) corresponding rennet gels, 5) acid gel from heated milk, and 6) stirred acid gel from
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39 387 heated milk. Authors reported that 16 000 peptides were clearly sequenced and identified and the
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41 388 dairy structure had only a little influence on the localization of cleavage sites as regards with protein
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43 389 sequences. However structure strongly impacted the number of identified peptides more specifically
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45 390 for rennet gel that led to three-fold less identified peptides. As for the previous study effect was
46
47 391 attributed to the the more important degree of dilution in the stomach by digestive secretions plus a
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49 392 longer gastric retention. These results led to an important conclusion: more than proteolysis
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51 393 mechanisms the dairy matrix structure strongly impact dairy protein digestion kinetics.

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53 394 From data collected in minipigs towards the digestion of different dairy matrices, Le
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55 395 Feunteun *et al.* developed a mathematical model for dairy protein digestion (Le Feunteun *et al.*,
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57 396 2014). For collecting data they used six 18-month-old female adult mini-pigs and six distinct dairy
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59 397 matrices (same as those described in the paragraph above) with identical composition based on a
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398 skimmed milk powder. Model was therefore developed to correspond to *in vivo* data. To summarize
399 the collected data clearly showed that dairy matrix structures impact protein digestion kinetics, and
400 the great differences between amino acid absorption kinetics can be entirely understood by
401 considering the behaviour of dairy matrices in the stomach. Thus the raw rennet gel clearly
402 exhibited the more sustained and low appearance of plasma amino acids while raw milk led to the

1
2 403 highest peak of plasma amino acids only after 20-30 minutes digestion. Other matrices had
3 404 intermediary behavior but the tendency for amino acid absorption appears to be in decreasing order:
4 405 milks > acid gels > rennet gels. These results well emphasize that it may exist slow and rapid dairy
5 406 protein only according to matrix structure, not composition, as was demonstrated earlier with whey
6 407 *versus* casein (Boirie et al., 1997).

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8 408 Although composition of infant formula has been significantly improved during the last
9 409 decade, major differences with the composition and structure of breast milk still remain and might
10 410 affect nutrient digestion and gut biology. The incorporation of dairy fat in infant formulas could
11 411 modify their physiological impacts by making their composition closer to that of human milk. The
12 412 effect of milk fat and milk fat globule membrane (MFGM) fragments in infant formulas on gut
13 413 digestion, mucosal immunity and microbiota composition was evaluated (Le Huërou-Luron et al.,
14 414 2016). Three formulas containing either (1) vegetable lipids stabilized only by proteins (V-P), (2)
15 415 vegetable lipids stabilized by a mixture of proteins and MFGM fragments (V-M) and (3) a mixture
16 416 of milk and vegetable lipids stabilized by a mixture of proteins and MFGM fragments (M-M) were
17 417 automatically distributed to 42 newborn piglets until slaughter at postnatal day 7 or 28, and
18 418 compared to a fourth group of sow's suckling piglets used as a breast-fed reference. At both
19 419 postnatal day, casein and β -lactoglobulin digestion was reduced in M-M proximal jejunum and
20 420 ileum contents compared to V-P and V-M ones leading to more numerous β -casein peptides in M-M
21 421 contents. In conclusion, the incorporation of both milk fat and MFGM fragments in infant formula
22 422 modifies protein digestion.

23 423

24 424 Human studies

25 425 All collected human studies were led in healthy adults; but only one study *stricto sensu* studied the
26 426 effect of dairy matrices - as eaten - on protein metabolism in humans (Gaudichon et al., 1995).
27 427 Other studies used casein or whey fractions (Boutrou et al., 2013; Churchward-Venne et al., 2015;
28 428 Gaudichon et al., 1999), or compared cottage cheese with eggs (Nuttall and Gannon, 1990).

29 429 The bioavailability study by Gaudichon et al. pointed out to the role of dairy matrices on
30 430 protein digestibility in sixteen healthy adults (average age of 30 years) by using radiolabelled milk
31 431 and yogurt (from the same milk) with ^{15}N (Gaudichon et al., 1995). Both products significantly
32 432 stimulated endogenous nitrogen secretion 20-60 minutes after consumption, and upon four hours
33 433 endogenous nitrogen flow rate did not differ between both matrices. An expected high level of
34 434 proteolysis was also observed for both matrices. However the yogurt led to delayed gastric
35 435 emptying as regards with exogenous nitrogen flow rate as compared to milk. Otherwise when
36 436 evaluating the net gastro-jejunal exogenous nitrogen absorption there was not significant difference
37 437 between milk (57%) and yogurt (51%). Finally, concerning non-protein nitrogen, the measured flow

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2 438 rate for yogurt tended to be higher to that of milk between 40-100 minutes after consumption. As
3 439 shown with mini-pigs it seems that the stomach step plays an important role in controlling the
4 440 delivering of protein in the small intestine and for the further absorption of amino acids with
5 441 potential application for protein anabolism especially in elderly populations.

6 442 The study by Boutrou *et al.* does not relate to complex dairy matrices as usually consumed
7 443 but casein and whey proteins (Boutrou *et al.*, 2013). However, since these two milk proteins have
8 444 different structures, the results obtained give interesting information on the peptide profiles and the
9 445 presence of bioactive peptides after digestion at the jejunal level according to these two protein
10 446 structures. The study has been led during 6 hours post-prandially in thirteen healthy adults (18-40
11 447 years old) consuming 30 g of either radiolabelled (^{15}N) casein or lactoserum. First, the recovery of
12 448 dietary nitrogen indicated that 50% and 60% of ingested nitrogen had already been absorbed after 6
13 449 hours at the jejunal level in WP and casein groups, respectively. Then the jejunal peak of exogenous
14 450 nitrogen after whey consumption was largely higher than that of casein at 0.5-1 hour after
15 451 consumption; but casein showed a more regular and lower nitrogen profile in agreement with
16 452 previous results about rapid *versus* slow protein (Boirie *et al.*, 1997). And the peak of jejunal
17 453 peptides was around 0.5-2.5 hours for casein and one hour for whey. Casein was a more important
18 454 peptide precursor at jejunal level with 356 identified and sequenced peptides against 146 for whey.
19 455 Casein peptides also presented varied biological activities and were of average size, *i.e.*, 750-1050
20 456 KDa during 6 hours while whey presented during 3 hours peptides with larger size, *i.e.*, 1050-1800
21 457 KDa. Moreover, the quantities of casomorphins β (caseins β 57, 58, 59 and 60-66) and casein β
22 458 108-113 released were sufficient to obtain a biological action of these peptides (*i.e.*, respectively
23 459 opioid and antihypertensive).

24 460 The second study by Gaudichon *et al.* did not compare different dairy matrices but it
25 461 deserves to dwell on it because it indirectly evaluates the "matrix effect" of milk by comparing the
26 462 protein digestion of radiolabeled casein alone and in the presence of sucrose or milk lipids
27 463 (Gaudichon *et al.*, 1999). Thus, 25 healthy male and female subjects (mean age of 29 years)
28 464 consumed three different diets, each containing 30 g of defatted milk proteins labeled with ^{15}N : 1)
29 465 milk protein alone; 2) milk proteins + sucrose (100 g); and 3) dairy protein + 43 g of dairy lipids
30 466 (from 36 g of butter and 46 g of cream). Sucrose clearly slowed down protein absorption on longer
31 467 time than lipids but in the end ileal digestion did not differ between the three diets (around 95%).
32 468 Sucrose also significantly reduced post-prandial transfer of nitrogen from milk to urea but not lipids
33 469 again. Secondly the net post-prandial milk protein utilization (8 hours after meals) was significantly
34 470 higher with sucrose (85%) compared to protein alone or with lipids (80%). According to authors
35 471 « This study shows that energy nutrients do not affect the nitrogen absorption but modify the
36 472 metabolic utilization of dietary protein in the phase of nitrogen gain » (Gaudichon *et al.*, 1999).

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2 473 In the following study, Churchward-Venne *et al.* have hypothesized that the digestion and
3 474 kinetics of protein absorption and the subsequent response of muscle protein synthesis after
4 475 ingestion of micellar casein is modulated by the dairy matrix (Churchward-Venne *et al.*, 2015).
5 476 Their assumption or hypothesis was based on the well-known fact that the slow digestion and slow
6 477 kinetics of amino acid uptake from isolated micellar casein are believed to be responsible for the
7 478 relatively lower postprandial response of muscle protein synthesis compared to those obtained with
8 479 more rapidly digested proteins such as isolated whey. In this study subjects were older than in
9 480 previous one (average age of 71 years) and consumed 600 mL casein with ¹³C leucine in either
10 481 bovine milk serum or water. The reconstructed dairy matrix (casein + bovine milk serum) did not
11 482 significantly modulate the post-prandial rate of myofibrillar protein synthesis compared to isolated
12 483 casein; and there was no significant difference for ¹³C phenylalanine enrichment of muscular
13 484 proteins. Therefore if casein of a given dairy matrix delays digestion and absorption of protein it
14 485 does not modulate post-prandial muscular protein synthesis as compared to isolated micellar casein.
15 486 More generally authors concluded that « These findings demonstrate that the anabolic response to
16 487 micellar casein protein is not compromised or enhanced by the milk matrix » (Churchward-Venne
17 488 *et al.*, 2015).

18 489 Although the following study is quite ancient and does not compare two different dairy
19 490 matrices, but that of cottage cheese and egg white, its results are interesting to analyze because both
20 491 products have quite closed protein (12.1 *versus* 10.6%, respectively), carbohydrates (2.2 *versus*
21 492 0.9%) and lipids (3.9 *versus* 0.2%) compositions, but with different matrices (Nuttall and Gannon,
22 493 1990). The study was led in seven healthy adults (average of 30 years) consuming either 480 mL
23 494 water or 50 g cottage cheese or egg white. After 8 hours the area under curve for plasma nitrogen
24 495 from amino acids and urea was lower by 50% for egg white compared to cottage cheese, notably
25 496 between 0-4 days. This was associated with a 50% lower protein conversion in urea. Overall 70% of
26 497 cottage cheese might explain the urea formation and only 47% for egg white. Finally cottage cheese
27 498 exhibited a better protein digestibility.

28 499

29 500 Conclusions on protein fraction

30 501 First of all, it should be noted that there are, in the end, very few studies comparing the digestive
31 502 evolution, more particularly that of proteins, of dairy products as consumed by man and presenting
32 503 different matrices. From the synthesis of *in vitro*, animals and human studies, it is difficult to draw
33 504 firm conclusions about the influence of the dairy matrix on digestibility, bio-accessibility,
34 505 bioavailability and nutritional effects of the protein fraction. However, some conclusions, although
35 506 still partial, can be proposed in relation to the studies described above (see also Table 1):
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2 508 *Milks versus acid gels versus rennet gels (mini-pigs)*

3 509 In pigs, based on dairy matrices of the same composition but differing in their internal structures,
4 510 the large differences in the kinetics of absorption of amino acids can be fully understood by
5 511 considering behavior of dairy matrices in the stomach. Compared to gelling by rennet, acid gelling
6 512 results in a higher peak of duodenal proteins as well as for amino acids, suggesting resistance of
7 513 rennet gel to gastric digestion; but the differences are leveled at the jejunal level. More generally,
8 514 gelation slows gastric emptying and subsequent absorption of amino acids, and decreases their
9 515 bioavailability in peripheral blood. Moreover, the structure of dairy matrices strongly affects the
10 516 kinetics of digestion of proteins rather than the mechanisms of proteolysis *per se*. Another main
11 517 conclusion is that both microstructure and macrostructure are affected by heating and gelification
12 518 processes, respectively, and that both impact differently proteolysis steps, microstructure acting
13 519 more on hydrolysis and macrostructure more on amino acid absorption and bioavailability.
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23 521 *Cheeses (in vitro)*

24 522 Depending on their texture, cheeses such as Camembert (soft cheese), blue-veined cheese, young
25 523 and aged Cheddar cheese, and Mozzarella (elastic structure) have different disintegration and
26 524 digestion patterns during gastric digestion. Thus, the disintegration at the end of gastric digestion is
27 525 higher when the initial hardness, cohesion and chewability are lower. Thus, Mozzarella, with higher
28 526 initial cohesion and hardness values, is disintegrated slowly and continuously during gastric
29 527 digestion. At the end of gastric digestion, elastic-type cheeses (Mozzarella) are about three times
30 528 less disintegrated in comparison with ripened soft cheeses with a high fat content (Camembert, aged
31 529 Cheddar); and the release of peptides/proteins, in agreement with the disintegration kinetics, is
32 530 globally two times lower. Textural properties and structural characteristics such as protein matrix
33 531 density and fat distribution also affect degradation during the duodenal phase.
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40 532 Concerning Norwegian cheeses Gamalost (grained cheese) *versus* Norvegia (firm cheese)
41 533 more peptides are detected in digested Gamalost compared to Norvegia cheese. Moreover, most of
42 534 the Gamalost peptides are derived from β -caseins, some from casein α 1, and very little from casein
43 535 α 2 and casein κ . Gamalost and Norvegia cheeses therefore have different protein digestibility
44 536 profiles, underlining an important "matrix" effect.
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49 537 If we can attempt a first general conclusion on the influence of cheese matrices on
50 538 proteolysis, it would appear that more cohesive, compact, elastic and structured cheeses disintegrate
51 539 less rapidly during digestion, especially at the gastric level, resulting in slower proteolysis. This
52 540 result would be in line with what has been observed with breads, *i.e.*, those with more dense crumb
53 541 give more time-spreading glycemic responses and have lower glycemic indexes (Burton and
54 542 Lightowler, 2006; Saulnier and Micard, 2012).
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Milks versus yogurts versus fermented milks (in vitro, mini-pigs and humans)

The digestion of casein begins during the gastric phase and appears slower for pasteurized milk than for yogurt. At the end of duodenal digestion, no intact casein is present in the different dairy matrices, while small bands of whey proteins are still visible for pasteurized milk and yoghurt compared to sterilized milk. The release of free amino acids during the duodenal phase varies according to their nature (acid, basic, neutral or hydrophobic) and appears to be governed by the specificity of the enzymes. For both types of matrix, the hydrophobic amino acids are released in significantly greater amounts than other types of amino acids. But overall, total protein digestibility seems to be identical between milk and yoghurt even though yogurt digestion results in different peptide profiles. Whether for milk or yogurt, digestion of β -casein gives a very significant number of peptides covering almost the entire sequence of this protein. It is believed that lactic fermentation is responsible for the formation of new peptides during gastrointestinal digestion. Compared to milk, yoghurt could also delay intestinal nitrogen delivery, but not final absorption. Concerning fermented milks, there is little data: fermentation of milk affects the release of certain amino acids during *in vitro* digestion. Proteolysis during fermentation can lead to the formation of new peptides during gastrointestinal digestion.

In mini-pigs, milk and yogurt proteins are also highly digestible (93%); and the kinetics of exogenous nitrogen delivery in the intestine is highly correlated with that of exogenous nitrogen uptake for milk and yoghurt. Gastric emptying also appears to be a major factor for controlling the kinetics of nitrogen absorption, the higher viscosity of yogurt allowing a more regular release of nitrogen into intestine leading to a more gradual absorption and distribution of dietary nitrogenous compounds within organism. Finally, in man fermentation only modifies the rate of gastric emptying of nitrogen (delayed with yoghurt) and does not affect the level of hydrolysis of milk and yoghurt made from the same milk, stimulation of endogenous nitrogen and digestibility rate. Whether in the mini-pig or man stomach seems to play a very important role in controlling the emptying of more or less hydrolyzed proteins in the small intestine.

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The lipid fraction*In vitro* studies*Milks, yogurts and derived products*

Very few studies have investigated *in vitro* lipolysis of dairy products with different matrix. The first study was led with fresh raw bovine milk submitted to *in vitro* gastrointestinal digestion (Gallier et al., 2012). Briefly fat globules were stable in acidic conditions within stomach due to phospholipids. Then at intestinal level, lipolysis products (released from the hydrolysis of

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2 578 triglyceride core) destabilized fat globules and provoked their coalescence or aggregation.
3 579 Thereafter lipolysis products accumulated to the surface of fat globules forming a lamellar phase.
4 580 Their solubilisation by bile salts led to the formation of disk-shapes micelles. Finally the free fatty
5 581 acid release after one hour of gastric digestion and two hours of intestinal digestion followed a
6 582 logarithmic-type curve (without lag phase), and reached 114 μmol free fatty acids/mL of
7 583 subsample.

8 584 Initially the study by Kopf-Bolanz *et al.* was dedicated to validate an *in vitro* digestion
9 585 system for investigating macronutrient decomposition in human; and homogenized and pasteurized
10 586 milk was chosen as an example of a complex food matrix (Kopf-Bolanz *et al.*, 2012). Although
11 587 there is no comparison between different dairy matrices, the results on digestion of the lipid fraction
12 588 deserve to be reported: first, the digestion process increased free fatty acid content from 1,04 to 62,8
13 589 mmol/L, *i.e.*, 41% of total milk fat content (however, assuming that all triglycerides present in
14 590 whole milk were hydrolyzed into two free fatty acids and one monoacylglycerol, this value
15 591 corresponded to 100% of the triglycerides); second, as regards with fatty acid distribution, more
16 592 short-chain fatty acids (*i.e.*, around 11%) were released after digestion compared to relative total
17 593 fatty acid content before digestion (*i.e.*, around 6%).

18 594 Subsequently, in the study of Tunick *et al.* (Tunick *et al.*, 2016), described above for protein
19 595 digestion, authors investigated the role of milk processing treatments on proteins and lipids
20 596 digestion. Homogenization increases free fatty acids release attributed to the increase surface area
21 597 improving lipase efficiency (Armand *et al.*, 1992). This was confirmed by the confocal microscopy
22 598 images during *in vitro* digestion showing smaller lipid droplets for homogenized HTST-pasteurized
23 599 whole milks. The fatty acids profiles were also determined but their release was not affected by the
24 600 processing treatments used in this study.

25 601 Finally, the *in vitro* study by Ye *et al.* provides interesting evidence on the role of the matrix
26 602 effect of milk by comparing the digestion of the fat globules of a raw native milk and a recombinant
27 603 milk from skim milk mixed with 4 % of anhydrous milk fat (Ye *et al.*, 2010). The fat globules in
28 604 raw milk are coated by natural milk fat globule membrane that is composed predominantly of
29 605 phospholipids and milk fat globule membrane proteins whereas the fat globules in recombined milk
30 606 are coated by casein micelles and whey protein. Overall there was a lower lipolysis in the raw milk
31 607 than in the recombined milk. Authors suggested that « this is probably because of the inhibitory
32 608 effect of components in the milk fat globule membrane on the activity of lipase » (Ye *et al.*, 2010).
33 609 Thus, the conversion of fat globules with their membrane (as in raw milk) into fat globules with
34 610 superficial layers of casein and serum proteins (as in recombinant milk) increases lipolysis rate of
35 611 dairy fat, involving an inhibitory effect of the membranes of the fat globules on the hydrolytic
36 612 action of the pancreatic lipase. In this study authors also reported that the rate and degree of

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2 613 lipolysis appears not to be influenced by the initial size of the fat globules. Finally after 5 hours of
3 614 hydrolysis the extent of fatty acid release was equivalent for both milks.
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6 616 *Cheeses*

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8 617 The casein-calcium interactions are mainly responsible for the structuring of the protein network in
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10 618 the cheese, and therefore for its textural properties (Lucey and Fox, 1993; Lucey et al., 2003). Thus,
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12 619 in the most recent study Cheddar cheeses with different levels of calcium were made to establish
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14 620 how this compound impacts the *in vitro* digestion of cheese lipid fraction: 1) cheese control (25.0
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16 621 mg calcium/g protein); 2) high-calcium cheese (29.6 mg calcium/g protein); and 3) very high
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18 622 calcium cheese (46.4 mg calcium/g protein) (Ayala-Bribiesca et al., 2016). As expected, the
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20 623 hardness of the cheese increases with the calcium content, resulting in a slower disintegration
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22 624 during digestion. However, despite more rapid disintegration, control cheese has the slowest
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24 625 lipolysis progression. This can be attributed to the fact that calcium increases lipolysis rates, an
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26 626 effect that can be explained by the depletion of fatty acids at the lipid-water interface. The final
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28 627 lipolysis rates after 300 min of digestion were 73.6%, 77.9%, and 72.5% for the control, high-
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30 628 calcium, and very high-calcium cheeses, respectively, with a statistical difference between the last
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32 629 two ($P = 0.0148$). Finally, this study suggests that lipolysis depends on the calcium content and the
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34 630 matrix modulating the access of enzymes to their substrates. To further understand the role of
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36 631 calcium on dairy fat lipolysis, Cheddar cheese were prepared with anhydrous milk fat with low
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38 632 (olein: unsaturated fatty acids and short chains fatty acids) or high (stearin: triacylglycerol and long
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40 633 chain fatty acids) melting point fatty acids (Ayala-Bribiesca et al., 2017). A control was also
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42 634 prepared with melted butter. For each Cheddar cheese prepared, two levels of calcium were also
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44 635 studied: regular (7,136 $\mu\text{g/g}$ of cheese) or high (9,466 $\mu\text{g/g}$ of cheese). The addition of calcium had
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46 636 no impact on cheese hardness at the opposite of the type of anhydrous milk fat. The cheese prepared
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48 637 with stearin anhydrous milk fat was 50% harder when the texture was measured at 22°C. A large
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50 638 proportion of the control and olein anhydrous milk fat was liquid at the measurement temperature as
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52 639 shown in the calorimetry thermogram. When measured at 37°C, most of the fat is liquid for all
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54 640 cheeses and no significant difference in cheese hardness was observed. The stearin cheese was still
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56 641 higher and retained its shape after compression mainly attributed to the residual solid fat content.
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58 642 This had a significant impact on cheese disintegration where stearin cheese were less disintegrated
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60 643 during gastric digestion compared to the other cheese matrices. In addition, no effect of calcium
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62 644 was found for the disintegration. The lipolysis was significantly modulated by the type of
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64 645 anhydrous milk fat and the calcium concentration. For the regular calcium cheeses, the stearin
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66 646 cheese had a lower lipolysis at the end of the duodenal digestion (below 50%) compared to the
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68 647 other cheeses. Short- and medium-chain fatty acids are more soluble and easily cleaved by lipase

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2 648 (Mu and Porsgaard, 2005) favoring an increased fatty acid release for the control and olein
3 649 anhydrous milk fat cheeses, both rich in short- and medium fatty acids (Ayala-Bribiesca et al.,
4 650 2017). For high calcium cheeses, the kinetics of lipolysis was significantly different during
5 651 digestion. Within the first 15 min, lipolysis was significantly increased for olein cheese compared to
6 652 stearin and was maintained until 240 min. At the end of the duodenal digestion, lipolysis increased
7 653 for the stearin cheese to reach a similar value than the other cheeses.

8 654 Lamothe *et al.* added that although composition factors (total lipid and calcium
9 655 concentrations) are known to influence the lipolysis rate, the characteristics of the cheese matrix are
10 656 also an important factor affecting the release of fatty acids, in particular *via* the restriction of
11 657 enzyme and fatty acids diffusion (Lamothe et al., 2012). In their study, lipids are released from
12 658 cheese matrices differently during the gastric phase, with aged Cheddar releasing fats faster than
13 659 mild Cheddar and Mozzarella, than light Cheddar, but the release rates are the same after 4 hours of
14 660 gastroduodenal digestion for all cheeses (Lamothe et al., 2012). Authors explained that the stronger,
15 661 denser and more cohesive matrix structure of light Cheddar could explain the lower rate of lipid
16 662 digestion relative to other cheeses during gastric digestion. In addition free oil was strongly
17 663 correlated with cheese disintegration during the different stages of cheese digestion. However,
18 664 concerning free fatty acid release following lipid hydrolysis during the duodenal digestion the
19 665 higher moisture and lower fat content of light Cheddar cheese make it easier for the enzymes to
20 666 diffuse within the cheese matrix and react with fat droplets. On the contrary, according to authors
21 667 the much lower degree of cohesion of aged Cheddar could promote matrix disruption into fine
22 668 particles for which the greater firmness and lower porosity could contribute to the resistance of
23 669 lipids to lipase attack. Then the greater extent of matrix disruption for aged Cheddar probably
24 670 explains why its rate and extent of free fatty acids production were slightly greater after 60 min of
25 671 duodenal digestion compared to mild Cheddar.

26 672 Therefore, for cheeses, greater fatty acid release could not be related to faster matrix
27 673 disintegration, suggesting that the lipid droplet size dispersion was more important than matrix
28 674 breakdown was for the modulation of lipid digestion kinetics (Lamothe et al., 2017).

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30 676 *Infant formulas*

31 677 The effect of homogenization on gastric lipolysis was studied by investigating the semi-dynamic
32 678 gastric *in vitro* digestion of three matrices (three model infant formulas): a standardized milk
33 679 emulsion containing native milk fat globules referred to as minimally-processed emulsion, and two
34 680 processed model infant formulas (homogenized or homogenized/pasteurized) (Bourlieu et al.,
35 681 2015). Gastric conditions mimicked those reported in newborns. The minimally-processed emulsion
36 682 was lipolyzed and proteolyzed slower than processed formulas. The difference in initial structure

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2 683 persisted during digestion. The surface of the droplets was the key parameter to control gastric
3 684 lipolysis kinetics; it ranged from 1.81 m²/g of lipid for minimally-processed formula to 31.90 m²/g
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5 685 of lipid for the most processed one. The pattern of released fatty acids and proteolysis by faster
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7 686 hydrolysis of adsorbed proteins was also significantly affected by homogenization.

8 687

9 688 Animal study

11 689 The objective of the study by Fruekilde *et al.* was to examine in the rat for the effects of the
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13 690 physicochemical properties of dairy products on lipid absorption at the lymphatic level (Fruekilde
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15 691 and Hoy, 2004). Although with very different fat contents (21 to 80%), the five dairy products
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17 692 tested (cream cheese, cream, sour cream, butter and melted butter) differ little in terms of their fatty
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19 693 acid composition. Administration of cream and sour cream leads to a faster absorption of the lipid
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21 694 fraction at the lymphatic level than with cream cheese, butter and melted butter, and a higher
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23 695 cumulative absorption after 8 hours. The lymphatic absorption of the fat after administration of the
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25 696 cream cheese is similar to the absorption after consumption of butter and melted butter for up to 4
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27 697 hours; then it increases to a level between that obtained with the consumption in the rat of cream or
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29 698 sour cream, and of butter or melted butter. Overall, these results demonstrate different profiles of
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31 699 lymphatic absorption of dairy fat by their physicochemical properties. Because the fatty acid
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33 700 composition of dairy products differs only slightly, this indicates that the viscosity, the type of
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35 701 emulsion, the particle size and possibly also the protein content, influence the digestion and
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37 702 absorption of the dairy fat, and probably affects the lipemic response.

38 703

39 704 Human studies

41 705 Human studies comparing the effect on lipemia of different ready-to-eat usual dairy matrices are not
42
43 706 so numerous. In the ten selected studies milk, fermented milk, butter, cheese, cream and yogurt
44
45 707 have been tested against lipemia mainly in healthy adults, but also in subjects with metabolic
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47 708 syndrome, type 2 diabetes, and mildly hypercholesterolemic.

49 709 In the first and recent randomized and crossover study the impact of the cheese matrix on
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51 710 postprandial lipemia in healthy humans has been evaluated (Drouin-Chartier *et al.*, 2017). Subjects
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53 711 consumed 33 g fat from a firm cheese (young cheddar), a soft cream cheese (cream cheese), or
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55 712 butter (control) incorporated into standardized meals that were matched for macronutrient content.
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57 713 After 4 h there was no difference in serum increases in triglyceride concentrations and in the
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59 714 triglyceride iAUC_{0-8 h} (area under curve) for the three dairy matrices. The most notable result was
60
715 the significantly higher triglyceride response induced by the cream cheese (change from baseline:
716 +44%) than those induced by butter (change from baseline: +24%) and cheddar cheese (change
717 from baseline: +16%). According to authors the fat globule organization within cheese matrices

1
2 718 may have modulated the postprandial lipid response. In effect homogenization of the cream cheese
3 719 increases the number and stability and decreases the size of lipid droplets. Thus, the diameter of a
4
5 720 lipid droplet from cream cheese is $\approx 0.5 \mu\text{m}$, approximately one-sixth the size of a cheddar cheese
6
7 721 lipid droplet ($\approx 3.0 \mu\text{m}$), increasing contact surface for lipase.

8
9 722 The second study has been led during 8 weeks in 153 Norwegians aged above 18 years with
10 723 ($n = 44$) and without ($n = 102$) metabolic syndrome (Nilsen et al., 2015). The subjects were divided
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12 724 into three groups: 1) Gamalost cheese (50 g/day) which is a traditional Norwegian skimmed milk
13 725 cheese naturally free of salt and fat, containing only 160 mg calcium/100 g, and which has a high
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15 726 protein content and a high amount of bioactive peptides; 2) Norvegia, a gouda-type cheese ($n = 50$)
16 727 (80 g/day) that contains 27% fat and 800 mg/100 g calcium; 3) control ($n = 50$). After intervention,
17
18 728 no increase in LDL and total cholesterol was observed in the 'cheese' groups compared to the
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20 729 control. However, a stratified analysis showed that subjects in the Norvegia group with the
21 730 metabolic syndrome showed significant reductions in total cholesterol at the end of the intervention
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23 731 compared to the control group (-0.70 mmol/L , $p = 0.013$) and also of triglycerides. In the Gamalost
24
25 732 group, subjects who had a high total cholesterol level at baseline showed a significant reduction in
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27 733 total cholesterol compared to control (-0.40 mmol/L , $p = 0.035$).

28 734 In the next randomized 3 x 2 week crossover study semi-hard cheese was compared to
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30 735 skimmed milk in 15 healthy young men (Soerensen et al., 2014). Focusing on the specific potential
31 736 effects of cheese calcium *versus* calcium in liquid milk, Soerensen *et al.* compared the effects of
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33 737 consumption of three isocaloric diets with similar lipid content and composition and varying in
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35 738 calcium intake. The calcium intakes in each of the three intervention periods were 400 mg/day
36 739 without dairy (control), or 1700 mg/day including 800 mg/day via dairy products, either as 670
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38 740 mL/day of skimmed milk, or 120 g/day of semi-hard cheese. Compared to the control diet, each of
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40 741 the two dairy-based diets limited total cholesterol and LDL cholesterol blood increases, but had no
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42 742 effect on triglycerides and HDL-cholesterol (no difference between the two dairy products), and
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44 743 resulted in a higher fecal lipid loss, which seems to be explained by their calcium content.
45
46 744 Therefore, although the effect on blood lipids do not differ substantially between milk and cheese
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48 745 consumption with similar calcium contents, results of this study support that calcium-rich dairy
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50 746 foods may be less atherogenic than their calcium-poor counterparts.

49 747 The following intervention study was performed in 159 moderately hypercholesterolemic
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51 748 French patients (without treatment) consuming twice daily 30 g of Camembert or 250 g of whole
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53 749 yogurt for 5 weeks (Schlienger et al., 2014). No significant difference for all lipid parameters was
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55 750 observed both between groups and after the intervention, *i.e.*, total cholesterol, HDL- and LDL-
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57 751 cholesterol and triglycerides.

1
2 752 In 2011, a randomized cross-sectional study compared in 49 healthy adult males and females
3 753 the effects of daily consumption of hard cheese (Samsø with 27% fat) for 6 weeks compared to
4 754 butter (bringing the same amount of lipids, accounting for 13% of energy intake) (Hjerpsted et al.,
5 755 2011). The cheese-based diet resulted in plasma LDL cholesterol concentrations 6.9% lower than
6 756 the butter-based diet. Given the composition of the diets, the authors ruled out that this may be due
7 757 to differences in calcium and protein concentrations between cheese and butter, and suggest
8 758 mechanisms related to fermentation and a "matrix effect" due to the dispersion of the fat globules in
9 759 the solid casein matrix of the cheese.

10 760 In another randomized cross-sectional study, healthy volunteers were subjected to a diet in
11 761 which 20% of their total energy intake was provided by dairy fat, either in the form of hard cheese,
12 762 milk or butter (Tholstrup et al., 2004). On the fourth day of each 3-week intervention period, an
13 763 acute postprandial test was performed with the test product. These tests revealed no difference in
14 764 the amount or profile of chylomicron fatty acids from cheese, milk or butter. However, a long-term
15 765 effect was observed: daily cheese consumption resulted in lower total cholesterol and LDL-
16 766 cholesterol than that of butter. These results are in agreement with the previous observation of a
17 767 lower total cholesterol level after consumption of cheese ('Jarlsberg', Swiss type) than butter
18 768 (Biong et al., 2004), both studies using diets where dairy fat constituted 20% of the energy intake.
19 769 At equal fat content the cheese can therefore be less hypercholesterolemic than butter (no
20 770 differences for triglycerides and HDL-cholesterol).

21 771 The last study with cheese has been led in diabetic subjects (Clemente et al., 2003). Test
22 772 meals containing 30 g of fat in the form of Mozzarella cheese or milk resulted in a peak plasma of
23 773 triglycerides earlier than the same amount of butterfat, although the cumulative absorption of
24 774 triglycerides on 6 hours (area under the curve) was similar in all groups. In addition, the rate of
25 775 gastric emptying is significantly faster with Mozzarella than with butter or milk. The authors
26 776 conclude that « while the physical structure of fat-rich foods has no major effect on postprandial
27 777 plasma triglyceride concentrations, it is able to influence the timing of triglyceride peak; gastric
28 778 emptying time does not play a major role in modulating the postprandial response of triglycerides
29 779 and glucose ».

30 780 In the last two studies fermented milk (Sanggaard et al., 2004) and yogurt (Rossouw et al.,
31 781 1981) were compared to milk towards lipemia. The study by Sanggaard *et al.* had the main
32 782 objective of comparing the effects of whole milk and fermented whole milk (with a mixture of
33 783 *Lactobacillus acidophilus* and *Lactococcus cremoris*) on postprandial lipid and carbohydrate
34 784 metabolism, rate of gastric emptying and appetite (Sanggaard et al., 2004). Lactose was added to
35 785 the fermented milk to equalize the lactose contents of both products. Fermented milk results in a
36 786 significantly slower rate of gastric emptying ($p < 0.001$), probably because of higher viscosity. In

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2 787 addition, fermented milk results in a greater increase and a more rapid decrease in the triglyceride
3 788 content in all lipoprotein fractions (LDL fractions, $p < 0.005$; other fractions HDL, VLDL and
4 789 chylomicrons, $p < 0.001$). The more prolonged and lower triglyceridemic response to milk
5 790 consumption may be due to coagulation of milk in the stomach. These effects were attributed to the
6 791 higher viscosity of the fermented milk and could therefore be applied to fresh cheeses. Finally,
7 792 Rossouw et al. conducted a short, 3-week randomized controlled study in 32 healthy adolescents
8 793 who consumed 2 liters of skimmed milk, yoghurt or whole milk cream per day (Rossouw et al.,
9 794 1981) in addition to their usual diet. Compared with pre-intervention values, plasma LDL-
10 795 cholesterol concentrations were reduced in the skimmed milk group but increased in the yoghurt
11 796 and cream groups.

12 797 Finally in a randomized controlled trial, eight hospitalized tube-fed preterm infants were
13 798 their own control to compare the gastric digestion of pasteurized human milk and of homogenized
14 799 pasteurized human milk. Pasteurized human milk was obtained from donors and, for half of it, was
15 800 homogenized by ultrasonication (de Oliveira et al., 2017). Over a six-day sequence, gastric aspirates
16 801 were collected twice a day, before and 35, 60 or 90 min after the start of pasteurized human milk or
17 802 homogenized pasteurized human milk ingestion. The impact of homogenization on pasteurized
18 803 human milk digestive kinetics and disintegration was tested using a general linear mixed model.
19 804 Results were expressed as means \pm SD. Homogenization led to a six-fold increase in the specific
20 805 surface ($P < 0.01$) of lipid droplets. The types of aggregates formed during digestion were different
21 806 between pasteurized human milk and homogenized pasteurized human milk, but the lipid fraction
22 807 kept its initial structure all over the gastric digestion (native globules in PHM vs. blend of droplets
23 808 in PHHM). Homogenization increased the gastric lipolysis level ($p < 0.01$), particularly at 35 and
24 809 60 min (22 and 24% higher for homogenized pasteurized human milk, respectively).
25 810 Homogenization of pasteurized human milk increased the gastric lipolysis level. This could be a
26 811 potential strategy to improve fat absorption, and thus growth and development in infants fed with
27 812 pasteurized human milk; however, its gastrointestinal tolerance needs to be further investigated.

28 813

29 814 Conclusions on the lipid fraction

30 815 Overall, these works show that differently structured dairy products, including cheeses, can result in
31 816 different profiles of postprandial lipemia. First, fatty acid release in the intestinal phase was much
32 817 faster when matrices were produced from homogenized milk (Lamothe et al., 2017). Second, the
33 818 composition of the cheeses, their viscosity and the presence of a matrix that is more or less solid, as
34 819 for a pressed cooked cheese compared to a fresh cheese, could affect the kinetics of digestion of the
35 820 lipids of cheeses. Indeed, the higher the viscosity of the food bolus, the longer the gastric emptying
36 821 time, which results in delaying the peak of postprandial triglyceridemia. This raises the question of

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2 822 the effects of the viscosity of different cheeses (soft fresh cheeses *versus* hard cheeses in particular)
3 823 on the metabolism of fat, especially when consumed as such in the French way, and not primarily in
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5 824 a melted form as in North America. On the basis of the *in vitro* and *in vivo* studies presented,
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7 825 additional conclusions can be proposed:

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10 827 *In vitro studies*

11 828 The comparison of a native milk with a recombined milk shows that the lipolysis rate of milk fat is
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13 829 increased when the fat globules with their membrane (as in raw milk) are converted into fat
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15 830 globules with superficial layers of casein and serum proteins (as in recombined milk), and that the
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17 831 rate and degree of lipolysis appears not to be influenced by the initial size of the fat globules; which
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19 832 could be attributed to changes in the physicochemical properties of fat globules during lipid
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21 833 hydrolysis.

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23 834 The casein-calcium interactions are mainly responsible for the structuring of the protein
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25 835 network in the cheese and the hardness of the cheeses thus increases with the calcium content,
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27 836 resulting in a slower disintegration during digestion; but although the final rate of lipolysis did not
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29 837 differ over three hours, the slowest lipolysis was observed with the fastest disintegrating cheese.
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31 838 Calcium thus influences both the structure of the cheese and the kinetics of lipolysis. Moreover,
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33 839 aged Cheddar (pressed) releases fat faster than mild Cheddar and Mozzarella (spun pasta of
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35 840 "elastic" type) but the final rate of release does not differ after four hours of digestion.

36 841

37 842 *In vivo studies*

38 843 In the rat, lymphatic absorption of lipids is faster for cream and sour cream compared to a cream
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40 844 cheese and butter. Viscosity, type of emulsion, particle size and protein content could play a role in
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42 845 the degree of lipolysis with identical composition of fatty acids. In humans, postprandial or multi-
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44 846 week intervention studies were performed either in healthy subjects or at risk for chronic diseases.
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46 847 Their results give indirect indications on "dairy matrices" effects according to the differences of
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48 848 lipemiae observed:

- 49 849 1) Hard cheeses cause LDL-cholesterol levels - and probably total cholesterol - to be
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51 850 significantly lower than butter; but this does not mean that the butter is
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53 851 hypercholesterolemic;
- 54 852 2) A semi-hard cheese does not give a different cholesterolemia than a skim milk;
- 55 853 3) Two different cheeses can have a different impact on total cholesterol;
- 56 854 4) A Camembert and a whole yogurt give similar cholesterolemia;
- 57 855 5) Compared to native milk, fermented milk results in a slower gastric emptying than liquid
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59 856 milk, as well as a higher increase and a faster decrease in triglyceride levels in all plasma

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2 857 lipoprotein fractions. These effects were attributed to the higher viscosity of the fermented
3 858 milk and could therefore be applied to fresh cheeses;

4
5 859 6) While the physical structure of the different dairy products does not appear to have a major
6 860 effect on plasma levels of postprandial triglycerides, it is still able to influence the time of
7 861 onset of the triglyceride peak, and gastric emptying time does not seem to play a major role
8 862 in modulating the triglyceridemic postprandial response.

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10 863
11 864

12 865 **Dairy food structure and calcium bioavailability**

13 866 *In vitro studies*

14 867 *In vitro* are measured only the bioaccessibility or the solubilisation of the calcium: the percentages
15 868 do not reflect in any way the actual bioavailability of calcium *in vivo*. However, they may give an
16 869 indication of its potential absorbability by the intestine, notably after dialysis.

17 870 In the study by Unal *et al.* several dairy products purchased from local Turkish supermarkets
18 871 were tested for the bioavailability of calcium *in vitro* (after dialysis): whole or skimmed milk, whole
19 872 or skimmed milk yogurt, fermented milks, and various cheeses (Turkish pickled white Cheese,
20 873 tulum cheese, cream cheese, kashari cheese and whey cheese) (Unal *et al.*, 2005). The products
21 874 differ in their acidity (percentage of lactic acid), ranging from 0.11% for whole milk to 1.78% for
22 875 Turkish pickled white cheese. The skimming of milk does not significantly alter the dialysability of
23 876 calcium, which is about 26-28%. For yogurts as well as for milks, no significant difference is
24 877 measured (28-33% bioavailability), leading the authors to conclude that the milk fat content has no
25 878 effect on bioavailability of calcium. On the other hand, significant differences are observed for
26 879 cheeses: from 14% for cream cheese to 32% for whey cheese. The study also shows that there is no
27 880 significant correlation between cheese acidity and calcium bioavailability. In accordance with the
28 881 literature (Allen, 1982), the authors attribute differences in bioavailability to the
29 882 phosphorus/calcium (P/Ca) ratio of cheeses, cream cheese having the highest ratio and whey cheese
30 883 the lowest. Finally, of all dairy products, skimmed milk yoghurt has the highest calcium
31 884 bioavailability (33%, $p < 0.05$). In another study the value obtained for cow's milk dialysability was
32 885 20%, a value lower than that calculated in the study by Unal *et al.* (Unal *et al.*, 2005). Shen *et al.*
33 886 also measured the dialysability of calcium from whole and skimmed cow's milk using *in vitro*
34 887 digestion (Shen *et al.*, 1995). The values obtained are of the same order of magnitude as the
35 888 previous studies, namely 19.6% for whole milk and 23.2% for skimmed milk, the difference not
36 889 being significant.

37 890 In the Cameroonian study of Ngounou *et al.* wild strains of *Streptococcus thermophilus* and
38 891 *Lactobacillus bulgaricus*, isolated from Cameroonian zebu milk, were used to ferment skimmed

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2 892 UHT milk (Ngounou et al., 2003). The determination of the soluble calcium in the fermented milks
3 893 obtained shows, whatever the bacterial strain, an increase in the solubility of this mineral. The
4 894 association of the two strains, while maintaining a high level of solubilization of the minerals,
5 895 appears less effective than the strains in individual cultivation. For example, with a single strain,
6 896 solubilization of calcium can reach almost 80%, whereas with two associated strains, the maximum
7 897 does not exceed 70%. The authors give no explanation for these differences and conclude that the
8 898 efficacy of these wild strains in solubilizing calcium justifies the interest of their biochemical
9 899 characterization in order to better define their contribution to the quality of the local fermented
10 900 milks.

11 901 In their review of 2014, Klobukowski *et al.* recalled that in an *in vitro* experiment carried
12 902 out in 2005 all the calcium in the milk was released in solution after digestion (Klobukowski et al.,
13 903 2014). Then, Klobukowski *et al.* briefly reported the results of a study conducted in 2005 but
14 904 unpublished with respect to the solubilization of milk calcium in the presence of cereal products.
15 905 Breakfasts based on milk alone or with cereal products were digested *in vitro*. Almost all calcium
16 906 from milk alone is released in solution. However, while corn and barley flakes and semolina
17 907 slightly decrease the amount of calcium measured in solution after digestion, wholemeal bread and
18 908 rolls retain about 50% of the calcium; which is a surprise for the authors as the rolls were made
19 909 from white flour. Then the authors reported the results of an earlier *in vitro* digestion study by
20 910 Skibniewska *et al.* wherein the calcium solubility of a commercial yoghurt was measured in the
21 911 presence or absence of cereal products (*i.e.*, corn flakes, wheat flakes, muesli, oat flakes, wheat and
22 912 rye bread slice, and slice of wholemeal bread) (Skibniewska et al., 2010). While the calcium
23 913 fraction released from the yogurt after digestion is about 67%, this fraction is always lower in the
24 914 presence of any cereal products, *i.e.*, from about 38 (oat flakes) to 65 % (slice of wheat and rye
25 915 bread). In a second experiment, 9 commercial yogurts with various cereal additives (unspecified)
26 916 were also digested *in vitro*: the released calcium fraction varies from 28.5 to 77.9% (Skibniewska et
27 917 al., 2010). These results led authors to wonder, in view of the general deficiency of calcium in the
28 918 diet in humans, whether or not dieticians should recommend mixing milk and cereal products.

29 919

30 920 ***Animal studies***

31 921 Calcium is by far the most studied mineral in animals. However, a significant number of studies
32 922 evaluated the bioavailability of calcium from fortified dairy products. These studies will therefore
33 923 not be discussed here unless a non-fortified control group was included in the experimental design.
34 924 Otherwise calcium bioavailability was also evaluated *via* bone criteria such as bone mineralization,
35 925 bone resistance and/or bone content.

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1
2 927 Dairy products

3 928 In the five following studies several distinct dairy products were compared in rats for their calcium
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5 929 bioavailability.

6 930 In the first study, the objective was to compare the biological availability (measured by
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8 931 apparent absorption and bone mineralization) of calcium in rats fed diets based on milk and various
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10 932 dairy products (semi-hard/hard cheese L'Envol made from skimmed milk with 4% fat, skimmed
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12 933 milk yoghurt and skimmed milk powder) containing various proportions of dairy proteins and
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14 934 calcium chemical forms (Delisle et al., 1995). The apparent absorption of calcium from cheese was
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16 935 higher ($\approx 35\%$) than from skimmed milk, yoghurt and skimmed milk powder ($\approx 13-18\%$) in young
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18 936 rats (6 weeks) whereas in adult rats (16 weeks) the apparent absorption of calcium is lower from
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20 937 cheese than from other dairy products. Nevertheless, the deposition of calcium at the level of the
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22 938 tibia and the femur and bone resistance were the same for all the diets.

23 939 The study by Buchowski *et al.* has compared the bioavailability of double-labeled calcium
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25 940 from different dairy matrices, *i.e.*, skimmed milk, yoghurt and fresh cheese curds (Buchowski et al.,
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27 941 1989). The radiolabeling was carried out intrinsically on goat milk with ^{45}Ca (injected into the
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29 942 jugular vein of the goat) and extrinsically (calcium solution added before the final incubation of 24
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31 943 hours at 4°C for the 3 products) with ^{47}Ca . First, the apparent absorption of ^{47}Ca is 69, 72 and 59%
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33 944 for milk, yogurt and cheese, respectively; and the apparent retention of ^{47}Ca is 61, 64 and 54%,
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35 945 respectively. In the tibia, there were no significant differences in retention (4.0-4.4%) and
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37 946 absorption (3.3-3.5%) of ^{47}Ca . Expressed as a percentage of the dose administered, absorption of
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39 947 ^{47}Ca was also strongly correlated with bone content in ^{47}Ca and ^{45}Ca ($R = 0.923$ for the correlation
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41 948 between the ^{47}Ca and ^{45}Ca content of the tibia). For each product tested, the extrinsic ^{47}Ca was
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43 949 absorbed similarly to the intrinsic ^{45}Ca . Finally, the percentage of radioactive dose retained in bone
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45 950 appears to be a valid indicator of the relative bioavailability of dietary calcium.

46 951 In the study by Ponerós *et al.* dried skimmed milk powder, freeze-dried Mozzarella cheese
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48 952 powder and calcium carbonate with or without 0.05% ascorbic acid were administered in rats
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50 953 (Ponerós and Erdman, 1988). Ascorbic acid has no influence on the bioavailability of calcium. The
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52 954 cheese had a higher calcium bioavailability (105%) - but not significant - compared to skimmed
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54 955 milk (95%). The calcium content of the tibia (calcium tibia/calcium ingested) was also not
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56 956 significantly different. It is surprising to find such high values, in particular greater than 100%.
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58 957 However, relative bioavailability was measured from the calcium tibia content according to the
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60 958 formula: tibia calcium (mg)/total consumed calcium (g); with a value of 100% attributed to calcium
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62 959 carbonate without ascorbic acid.

63 960 In the study by Kaup *et al.* several groups of rats have received different diets in which the
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65 961 calcium carbonate provides either the whole of the calcium or half which is then added half by

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2 962 calcium from 1) dried skimmed milk with or without lactic acid, 2) traditional yogurt (cultured with
3 963 *Streptococcus thermophilus* and *Lactobacillus bulgaricus*), 3) yoghurt termed acidophilus (cultured
4 964 with *S. thermophilus* and *L. acidophilus*) (Kaup et al., 1987). First, the ash bone content increased
5 965 with milk but not with yogurt; second, rats fed with milk with added lactic acid had thicker femurs
6 966 than with yogurts; thirdly, rats given yogurts and acidified milk had femurs with higher bending
7 967 moments (induced reaction when an external force or moment is applied to an element causing its
8 968 flexion) than rats given unacidified milk; finally bone strain decreased and the modulus of elasticity
9 969 increased as ingested calcium increased. Authors concluded that « the improved bone characteristics
10 970 in rats given milk, and particularly in those given acidified or cultured milk, in diets at constant
11 971 lactose content indicate that lactose is not the only contributor to the high bioavailability of calcium
12 972 in milk products and that lactic acid may also be a factor » (Kaup et al., 1987).

13 973 In the last study Dupuis *et al.* have studied the use of calcium from calcium carbonate
14 974 (lactose-free control group) and from three dairy matrices (skimmed milk, yogurt and processed
15 975 cheese) in growing (8 weeks) and ageing (14 months) rats (Dupuis et al., 1985). First, the calcium
16 976 of milk products was better utilized than in the control. During ageing and lactation, the milk and
17 977 yoghurt diets had the lowest bone exchanges while being higher in the control diet in the absence of
18 978 Ca utilization factors. Authors suggested that lactose may be responsible for differences in bone
19 979 exchanges of calcium within the milk products used.
20 980

21 981 Milks

22 982 In the four following studies calcium absorption and retention were measured in mice and rats. In
23 983 the first study carried out in mice, although not clearly explained, the milk used appears to be an
24 984 unprocessed whole cow's milk (Singh et al., 2007). The percentages of absorption and retention of
25 985 calcium are respectively 50 and 44%, values lower than those observed in the rat in the next studies.
26 986 Thus in the study by Toba *et al.* in 7-week-old rats the apparent uptake of calcium was
27 987 approximately 80% and only 45% in 12-week-old rats (Toba et al., 1998). The study by Brink *et al.*
28 988 was carried out in rats fed, inter alia, with a sterilized cow's milk in a diet supplemented with a
29 989 mixture with or without minerals and proteins in a first experiment, and with or without a mixture
30 990 of magnesium, sodium and potassium in a second experiment (Brink et al., 1992). In the first
31 991 experiment the absorption of calcium is 77% for normal milk and only 59% with the addition of a
32 992 mixture of minerals and proteins. In the second experiment, the absorption of calcium is similar and
33 993 is 74%, the addition of magnesium, sodium and potassium having only a small impact on this
34 994 absorption reduced to 70%. The last study compared the bioavailability of calcium in rats from
35 995 dried skim milk, white bread and wholemeal bread (Ranhotra et al., 1981). The bioavailability of

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2 996 calcium compared to that of the control group receiving calcium sulfate (bioavailability considered
3 997 100%) is higher with milk (113.3%) than with bread (99.4 and 104.4%).
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6 999 Yogurts

8 1000 The objective of the El-Gawad *et al.* study was to evaluate and compare the effect of cow's milk
9 1001 (3.2% fat) and various yogurts with (*Bifidobacterium lactis Bb-12* or *Bifidobacterium longum Bb-*
10 1002 *46*) or without probiotics on the bioavailability of calcium and bone mineralization in 48 male
11 1003 albino rats fed 45 days (El-Gawad *et al.*, 2014). The serum calcium levels did not differ
12 1004 significantly between the different dairy matrices. However, the highest apparent calcium
13 1005 absorption was observed in rats fed the yogurt-based diet cultured with *Bifidobacterium lactis Bb-*
14 1006 *12* (83%) followed by the yogurt cultured with *Bifidobacterium longum Bb-46* (82%), plain yogurt
15 1007 (78%) and cow's milk (76%). The authors suggest that peptides released by the degradation of
16 1008 caseins by digestive enzymes can promote the absorption of calcium by intestinal cells by
17 1009 preventing the precipitation of insoluble salts (Meisel *et al.*, 2003). Otherwise, bone mineral density
18 1010 of the femur is significantly higher with yogurt cultured with *Bifidobacterium lactis Bb-12*
19 1011 compared to other dairy matrices. Moreover, the rupture force of the femur does not differ
20 1012 according to the matrices. According to the authors, the suggested mechanisms of the positive effect
21 1013 of probiotics on mineral bioavailability and bone properties may include: 1) increased mineral
22 1014 solubility due to increased bacterial production of short-chain fatty acids, and 2) enlargement of the
23 1015 absorptive surface due to the promotion of enterocyte proliferation mediated by bacterial
24 1016 fermentation products (Perez-Conesa *et al.*, 2007). It is also possible that short-chain fatty acids can
25 1017 directly stimulate calcium absorption in the rat colon and that at least calcium could pass through
26 1018 the cell membrane more readily in the form of a less charged complex (acetate-calcium) *via* a
27 1019 passive route (Lutz and Scharrer, 1991).
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43 1021 Cheeses

44 1022 Different types of cheese have been tested in rats for their calcium bioavailability. First, based on
45 1023 studies in animals, Klobukowski *et al.* (Klobukowski *et al.*, 2014) recall in their review that the
46 1024 highest percentages of calcium apparent absorption and retention are observed for fresh cheese
47 1025 produced either by a conventional method (acid coagulation) (90.3% and 84.2%, respectively) or
48 1026 with the addition of probiotic *Lactobacillus plantarum* (90.5% and 86.3, respectively)
49 1027 (Klobukowski *et al.*, 1997b); this compared with the refined cheese (59.6-76.3% and 64.4-81.3%,
50 1028 respectively) produced by immersion in traditional (2% NaCl) or modified (NaCl and KCl solution
51 1029 at 1:1) brines or unrefined Ricotta-type cheese (57.4-60.5% and 52.0-52.2%, respectively) produced
52 1030 by the heat-acid protein coagulation method (Table 2) (Klobukowski *et al.*, 1997a). The following

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2 1031 study, carried out in the rat, tested the absorption of calcium from a white cheese supplemented or
3 1032 not with the probiotic *Lactobacillus plantarum* (Klobukowski et al., 2009). No significant
4 1033 differences in calcium retention percentages (≈ 84 - 86%) and calcium absorption ($\approx 90\%$) were
5 1034 observed for both cheeses.

8 1035 The objective of the study by Mora-Gutierrez et al. was to evaluate the apparent uptake and
9 1036 deposition of calcium (bone mineralization) in male rats fed diets containing Monterey Jack cheese
10 1037 (semi-hard cheese) based on bovine or caprine milk and enriched or not with milk calcium (Mora-
11 1038 Gutierrez et al., 2007). Five groups of rats were therefore given a control diet without casein or one
12 1039 of the following four experimental diets: (1) diet with bovine milk cheese; (2) diet with calcium-
13 1040 enriched bovine milk cheese; (3) diet with goat milk cheese; and (4) diet with calcium-enriched
14 1041 goat milk cheese. Significant differences are reported for calcium absorbability (referred to in this
15 1042 study as "calcium digestibility") in the following order: (4) $68\% > (3) 61\% > (2) 57\% > (1) 53\% >$
16 1043 (control) 41% . Significant increases are also observed for bone mineral content, bone mineral
17 1044 density and femoral bone rupture strength in the following order: (4) $> (3) > (2) > (1) > (control)$.
18 1045 The authors concluded that goat milk-based cheese characterized by high content of peptides from
19 1046 casein α_{s2} and β , enriched or not enriched with calcium, may be more protective against bone
20 1047 fragility than cheese from bovine milk, notably by increasing the absorption of calcium.

21 1048 Similar to the previous study, Kato *et al.* investigated the calcium bioavailability of a cheese,
22 1049 enriched or not with milk calcium, by examining bone strength and bone mineral density in growing
23 1050 male rats for 31 days (Kato et al., 2002). Unfortunately, the authors did not give percentages of
24 1051 absorbability. They reported the following results: compared to the control group "without cheese",
25 1052 the "cheese" groups had a significantly higher bone mineral density of the 4th lumbar vertebra;
26 1053 however, only the "calcium-enriched cheese" group had significantly greater strength and breaking
27 1054 strength of the femur than the control group; no significant difference in serum calcium
28 1055 concentration was observed between the three groups.

29 1056 Then, Delisle *et al.* have studied the bioavailability of calcium from three Cheddar (hard
30 1057 cheeses) differing in composition (31% fat $\pm 0.35\%$ whey proteins, and 7% fat) in ovariectomized
31 1058 rats (Delisle et al., 1997). The results interestingly showed that whey proteins do not affect the
32 1059 apparent absorption of calcium compared to the reference Cheddar at 31% fat (from 20% at 4 weeks
33 1060 to about 7% at 16 weeks), while a high level of lipids improves the apparent absorption of calcium
34 1061 and delays, although slightly, bone resorption (*i.e.*, reduction of bone loss between 8 and 16 weeks)
35 1062 with age compared to 7% fatty Cheddar. As in the previous study by Toba *et al.* (Toba et al., 1998),
36 1063 an "age" effect in favor of a decrease in apparent calcium absorption is highlighted.

37 1064 In the last study, based on the lack of information on the effect of cheese making,
38 1065 specifically the ripening time, on the bioavailability of calcium Buchowski and Miller compared the

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2 1066 bioavailability of calcium in rats from CaCl_2 (28 mM), CaCO_3 , milk (fresh and adjusted to pH 5.35
3 1067 - Cheddar pH) and Cheddar cheeses ($\approx 35\%$ fat) ripened for various periods - using double-labeled
4 1068 cheeses with ^{45}Ca and ^{47}Ca (Buchowski and Miller, 1990). The cheeses were made from pasteurized
5 1069 cow's milk. The bioavailability of calcium was determined by two methods: 1) the measured
6 1070 absorption based on the whole body radioactivity count; and 2) availability for bone metabolism
7 1071 assessed by bone radioactivity measurements. Calcium absorption varies from 72.8 (Cheddar at 0
8 1072 day ripening) to 81.2% (Cheddar as a slurry in a lactose solution). The ripening time of the cheese
9 1073 had no significant effect on calcium absorption. The calcium absorption from milks and CaCl_2 and
10 1074 CaCO_3 was similar around 75.7-79.3% ($p > 0.05$). There was no significant difference in the extent
11 1075 of calcium absorption in the tibia for all tested products (between 2.7 and 2.9%).
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19 1077 *Human studies*

20 1078 It is important to note that bioavailability studies in humans are generally rather old, probably due
21 1079 to (1) the development of more recent, cheaper and faster *in vitro* digestion studies, (2) the high cost
22 1080 of studies using stable isotopes, (3) non-authorization of radioisotopes, and (3) investment in time
23 1081 for this type of study.
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29 1083 Dairy products

30 1084 Two human studies have simultaneously compared milk, yogurt and cheese for their calcium
31 1085 fractional absorption. In the first one, four different dairy matrices were tested in healthy young
32 1086 Caucasian women, and no significant difference was observed between pasteurized and
33 1087 homogenized cow's milk (32.8%), Cheddar cheese (37.4%), yoghurt (24.2%) and processed cheese
34 1088 (33%) (Nickel et al., 1996). In the second study in healthy postmenopausal women, calcium
35 1089 absorption was not significantly different between whole milk (26.7%), chocolate milk (23.2%),
36 1090 yoghurt (made from whole milk with 2% fat) (25.4%), a milk substitute (prepared from milk and
37 1091 non-dairy products) (22.4%), a cheese (no information about its variety) (22.9%) and calcium
38 1092 carbonate (22.0%) (Recker et al., 1988).
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46 1094 Milks

47 1095 There are more studies comparing calcium fractional absorption in milk as compared with other
48 1096 foods or beverages (especially mineral waters), and also calcium carbonate.
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54 1098 *Milk vs mineral waters*

55 1099 Brandolini *et al.* recalled that it is well known that the intestinal availability of calcium from
56 1100 mineral waters rich in calcium is equivalent to that of milk (Brandolini et al., 2005). However, the

1 1101 effect of associated anions on urinary calcium losses has not been studied. Their study was carried
2 1102 out in cross-over in 37 healthy women over a period of 4 x 3 weeks and consuming the same
3 1103 amount of calcium from a SO₄-rich milk or mineral water (for anions SO₄²⁻). Urinary excretion of
4 1104 calcium is higher (+ 0.5 mmol/day) with water than with milk ($p < 0.001$), suggesting that calcium
5 1105 balance is better with milk intake than with SO₄-rich water. The acidogenic action of SO₄ would be
6 1106 responsible for this effect.

7 1107 In the study by Couzy *et al.* the bioavailability of milk calcium (UHT sterilized, 2.8% fat) is
8 1108 compared to that of mineral water in healthy young women (Couzy *et al.*, 1995). The fractional
9 1109 absorption of calcium from milk (25%) did not differ significantly from that of mineral water
10 1110 (23.8%).

11 1111 The study by Heaney and Dowell also compared the fractional absorption of milk calcium
12 1112 (no details given on technological treatments) and Italian mineral water in healthy women, and
13 1113 found no significant difference between both products (43.3 versus 47.5%, respectively) (Heaney
14 1114 and Dowell, 1994). However, differences in percent bioavailability between this study and the
15 1115 previous one may be surprising.

16 1116 Again, in the study by Halpen *et al.* the bioavailability of calcium (⁴⁵Ca) in milk (no data on
17 1117 technological treatments) was compared with calcium-rich mineral water in healthy adult males
18 1118 (Halpern *et al.*, 1991). The authors did not give percentages of absorption but report that the
19 1119 bioavailability of calcium from mineral water is generally as good as or better than that of milk.

20 1120 Again with a view to knowing whether calcium derived from mineral waters is better
21 1121 absorbed or not than from whole milk, Sheikh *et al.* have evaluated calcium absorption of different
22 1122 calcium salts dissolved in water with that of milk in healthy subjects (Sheikh *et al.*, 1987). The
23 1123 absorption of whole milk calcium (31%) was not significantly different from calcium salts.

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25 1125 *Milk vs other foods*

26 1126 Based on the assumption that the recommended intake of calcium may be difficult to achieve with
27 1127 only dairy products, Gonnelli *et al.* wanted to evaluate the calcium absorbability (marked with ⁴⁴Ca)
28 1128 of a new calcium-fortified orange beverage in cross-over and compare it with milk in healthy adults
29 1129 (Gonnelli *et al.*, 2007). The relative changes in calcium absorption from the two test drinks were
30 1130 similar (23.0% for milk and 20.9% for the orange beverage).

31 1131 Hitz *et al.* have compared the bioavailability of calcium from calcium carbonate with or
32 1132 without cholecalciferol, and from milk (no data on applied technological treatments) in young
33 1133 women (mean age 30 years) and a group of elderly people of both sexes (mean age of 66 years)
34 1134 (Hitz *et al.*, 2005). Each diet contains 1200 mg of calcium. Taking into account all participants,
35 1135 urinary excretion rates (mmol/day) of calcium were 4.41 for the placebo group (tablets), 5.17 for

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2 1136 milk, 5.83 for calcium carbonate and 6.06 for calcium carbonate supplemented with cholecalciferol.
3 1137 Compared to placebo, urinary excretion rates were all significantly different. The increase in
4 1138 calcium excretion with the "milk" diet is significant only for the elderly group. Using calcium
5 1139 excretion as an indirect measure of intestinal calcium absorption, the bioavailability of calcium
6 1140 from milk and calcium carbonate with or without cholecalciferol has been therefore demonstrated in
7 1141 healthy young and elderly individuals. Furthermore, the bioavailability of calcium in men and
8 1142 women over 60 years of age and healthy appears to be the same as in younger healthy women.

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10 1143 Zhao *et al.* studied the calcium bioavailability of soy milk fortified with calcium carbonate
11 1144 and that of cow's milk (without further details on the nature of the milk) in healthy young women
12 1145 (average age 23 years) (Zhao *et al.*, 2005). The fractional absorption of calcium did not differ
13 1146 between the two products (21.1% for soya milk and 21.7% for cow's milk). Similarly, Weaver *et al.*
14 1147 have studied the bioavailability of calcium from tofu and cow's milk in healthy pre-menopausal
15 1148 women of Caucasian and Asian origin with ^{44}Ca (Weaver *et al.*, 2002). In Caucasian women (whole
16 1149 milk), absorption of calcium is 54.8%, and 39.8% in Asian women (skimmed milk), a percentage
17 1150 not significantly different from that of tofu. Although studies by Zhao *et al.* and Weaver *et al.* have
18 1151 both used ^{44}Ca , their method of calculation differs a little to measure the absorption of calcium; but
19 1152 this does not explain the values of single to double reported (from 21.7% to 54.8%).

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21 1153 In the study by Griessen *et al.* the bioavailability of calcium was tested in normal male
22 1154 students but also lactase deficient (Griessen *et al.*, 1989). Three products were evaluated: 1) a
23 1155 reconstituted powdered milk containing lactose, 2) a reconstituted powdered milk where the lactose
24 1156 was replaced by glucose, and 3) water. The fractional absorption of calcium was 21.4% in normal
25 1157 subjects for reconstituted powdered milk containing lactose and nearly 30% in lactase-deficient
26 1158 subjects ($p < 0.05$).

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28 1159 Finally, in healthy adult women, Heaney *et al.* reported an absorbability of milk calcium (no
29 1160 data on technological treatments) of 31.7% versus 4.6% for spinach and 41% for kale (Heaney *et al.*,
30 1161 1990); and of 27.6% for milk and 5.1% for spinach in a previous study (Heaney *et al.*, 1988).

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32 1163 Yogurts

33 1164 Smith *et al.* studied the absorption of calcium from whole milk and yogurt in normal and lactose-
34 1165 deficient subjects (Smith *et al.*, 1985). Normal and deficient subjects equally absorbed calcium from
35 1166 milk (17.0 versus 19.8%, respectively) and yogurt (15.1 versus 20.6%, respectively).

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37 1168 Cheeses

38 1169 The first study did not measure directly calcium bioavailability, but rather the relative effectiveness
39 1170 of calcium supplementation from cheese or pills with or without vitamin D supplementation for

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2 1171 bone mass accrual during the rapid growth period in 195 healthy girls aged 10-12 years with dietary
3 1172 calcium intakes < 900 mg/day (Cheng et al., 2005). Four groups involved 1) 1000 mg calcium
4 1173 carbonate + 200 IU (5 µg) vitamin D daily; 2) 1000 mg calcium carbonate daily + vitamin D
5 1174 placebo; 3) 1000 mg calcium daily from supplemented dairy products (mainly low-fat cheese); and
6 1175 4) calcium placebo + vitamin D placebo identical to the effective pills. First, calcium
7 1176 supplementation with cheese resulted in a higher percentage change in cortical thickness of the tibia
8 1177 than did other three groups, and in higher whole-body bone mineral density than did placebo
9 1178 treatment. However, when controlling for growth velocity, these differences disappeared. Such
10 1179 results suggested that increasing calcium intake through consuming cheese would be more
11 1180 beneficial for cortical bone mass accrual than the consumption of tablets containing a similar
12 1181 amount of calcium.

13 1182 An indirect method of bioavailability measuring the parathyroid response to an oral load of
14 1183 calcium has also been used (Karkkainen et al., 1997). Thus, the comparison of nine adult women
15 1184 between Emmenthal cheese, milk, spinach, sesame seed and a mixture of medicated calcium salts
16 1185 (lactate, gluconate, carbonate) was in favor of the calcium of the cheese for a significant decrease in
17 1186 serum levels of intact parathyroid hormone, better than milk and equivalent to calcium salt, and
18 1187 much higher than calcium in spinach and sesame seeds (rich in oxalic or phytic acids) (Karkkainen
19 1188 et al., 1997). On the other hand, urinary calcium excretion was 141% ($p = 0.001$) with cheese, 107%
20 1189 ($p = 0.004$) with milk and 75% ($p = 0.02$) with calcium salt, above that of the control session. The
21 1190 authors concluded that their results indicate that fermented cheese may be a better dietary source of
22 1191 calcium than milk when the metabolic effects of a food are considered.

23 1192 Van Dokkum *et al.* studied the bioavailability of calcium (^{44}Ca) derived in particular from a
24 1193 fresh cheese (Petit Gervais with fruit), another fresh cheese (no further details on the product) and
25 1194 the same fresh cheese enriched with iron in healthy women (van Dokkum et al., 1996). The
26 1195 fractional absorption of calcium was 42.2, 37.7 and 38.8% ($p > 0.05$), respectively. The urinary
27 1196 excretion of calcium over 24 hours also did not differ.

28 1197 Finally, in the following study, the influence of the Emmenthal matrix (cooked pressed paste)
29 1198 on the bioavailability of calcium relative to that of calcium carbonate was evaluated; and on the
30 1199 basis of serum calcium, no significant difference was observed between both sources of calcium
31 1200 (Fardellone et al., 1993).

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33 1202 ***Conclusions on the calcium fraction***

34 1203 Specific conclusions on cheeses

35 1204 In 2008, the calcium bioavailability from cheeses has been reviewed and some first conclusions
36 1205 were already proposed (Guéguen, 2008) : 1) The actual absorption coefficient of calcium in milk

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2 1206 varies in adult between 25 and 35%; 2) Even if it is sometimes difficult to extrapolate to human the
3 1207 results obtained in rats or *in vitro*, at least it can be seen that calcium is as bioavailable, or even
4 1208 more, in cheeses than in milk; 3) Recent data in humans are few. They show that the calcium
5 1209 absorption of a cooked pressed cheese is significantly higher than that of calcium from various
6 1210 plants (Falcou and coll., 1988); and that the comparison between various dairy products and
7 1211 calcium carbonate confirmed the good intestinal absorbability of calcium in cheese, equivalent to
8 1212 that of carbonate (Recker et al., 1988).

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13 1213 Guéguen continued: « The absorbability of calcium in cheese thus seems good and
14 1214 equivalent to that of calcium in milk, despite the differences in constituents that could theoretically
15 1215 be unfavorable. Thus, the fact that most cheeses are poor in lactose could theoretically be
16 1216 unfavorable to calcium absorption, although the relative importance of this beneficial effect of
17 1217 lactose in a meal has been questioned (Gueguen and Pointillart, 2000). On the other hand, if the
18 1218 phosphopeptides are likely to increase the *in vivo* absorption of calcium (which is also debatable),
19 1219 their presence in a greater proportion in the cheese resulting from the degradation of the proteins
20 1220 during acidification and ripening should be a favorable factor. Finally, the risk of intestinal
21 1221 formation of insoluble calcium soaps from the long saturated fatty acids provided by non-lightened
22 1222 cheeses could be an unfavorable factor. In fact, we have shown that the possible formation of soaps
23 1223 occurs in the distal small intestine and thus insolubilizes calcium not previously absorbed and
24 1224 which, in any case, would no longer be so (Guéguen, 1992). It seems well established that saturated
25 1225 lipids do not interfere with the intestinal absorption of calcium, while excess calcium is likely to
26 1226 decrease absorption of free saturated fatty acids, which is beneficial » (pages 7-8) (Guéguen, 2008).

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37 1228 In 2017, based on this review, we propose new updated conclusions for all dairy products and
38 1229 calcium bioavailability:

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42 1231 *In vitro* studies

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44 1232 *In vitro* studies generally measure different parameters such as bioaccessibility, solubilization
45 1233 and/or dialysability *via* generally Caco2 cells, somehow measuring a form of *in vitro* absorption or
46 1234 bioavailability. The previously detailed studies provide the following potential conclusions:

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48 1235 1) The milk calcium is apparently almost completely solubilized *in vitro*, and its fermentation could
49 1236 further increase this solubilization;

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51 1237 2) The calcium dialysability of milk varies from 20 to 28% according to studies without effect of
52 1238 the fat content;

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54 1239 3) The calcium dialysability of yogurts varies from 28 to 33% without effect of the fat content or
55 1240 the acidity of the products;

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2 1241 4) The calcium dialysability of cheese varies from 14 to 32%, and may depend on the ratio of
3 1242 phosphorus/calcium (P/Ca);
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5 1243 5) In the presence of cereal products, solubilization of calcium from milk is reduced: while maize
6 1244 and barley flakes and wheat semolina slightly decrease the amount of calcium measured in solution
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8 1245 after digestion, wholemeal bread and small rolls retain about 50% of the calcium ;
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10 1246 6) While the calcium fraction released from the yogurt after digestion is about 67%, this fraction is
11 1247 always lower in the presence of any cereal products, *i.e.*, about 38 (oat flakes) to 65% (slice of
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13 1248 wheat and rye bread).

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15 1249

16 1250 Murine models

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18 1251 On the basis of studies in the adult rat the percentages of apparent absorption of calcium are
19 1252 summarized in Table 2. It should be noted, however, that the sometimes significant variability of
20 1253 the apparent absorption percentages for the same product must be attributed to the experimental
21 1254 conditions more than to true natural variability. The available studies therefore make it possible to
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23 1255 arrive at comparative comparisons and to propose conclusions or certain qualitative trends:

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26 1256 1) The apparent absorption of calcium from fermented milks is higher than that of a normal yogurt
27 1257 and that of milk; and the bone mineral density of the femur follows the same classification; also
28 1258 probiotics would increase the solubility of calcium, and increased bacterial production of short-
29 1259 chain fatty acids in the presence of probiotics would increase colonic absorption of calcium ;
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31 1260 2) Milk and yoghurt have similar calcium absorption but higher than cheese;
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33 1261 3) Absorption and retention of calcium is higher for fresh cheese compared to ripened or unripened
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35 1262 cheese;
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37 1263 4) The absorption of calcium from skimmed milk and Cheddar cheese is decreased with aging,
38 1264 which would correspond to a decrease in calcium requirements of the body;
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40 1265 5) The lipid content of cheese may increase calcium absorption and decrease bone resorption;
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42 1266 6) There is no significant difference in calcium absorption in the tibia for Cheddar cheese (with
43 1267 different ripening times) and fresh milk; and also for a skim milk, a yoghurt and a goat cheese curd;
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45 1268 7) The ripening time of a cheddar cheese has no significant effect on calcium absorption;
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47 1269 8) There is a correlation between calcium absorption and bone calcium content;
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49 1270 9) In addition to lactose (which may play a role in differences in calcium absorption between dairy
50 1271 products), lactic acid may play a role in the high bioavailability of calcium in some dairy products.
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52 1272 10) Some recent studies based on bone tests have confirmed the hypothesis formulated in 1992
53 1273 (Guéguen, 1992) on the equivalence of milk and cheese for the bone efficiency of calcium (Kato et
54 1274 al., 2002) (Delisle et al., 1995; Mora-Gutierrez et al., 2007).

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1276 Human studies

1277 The different studies carried out in healthy subjects, except for lactase deficient subjects, suggest
1278 the following conclusions:

1279 1) The absorption of milk calcium is between 17 and 55%, with the majority of studies giving
1280 values between 20 and 30%; which joins the conclusions of the previous review (Gueguen, 2005);

1281 2) The fractional absorption of milk calcium is similar to that of calcium from mineral water;

1282 3) Cheddar cheese has a fractional absorption of calcium (37%) slightly higher than milk (33%),
1283 processed cheese (33%) and yogurt (24%);

1284 4) The fractional absorption of yogurt calcium seems to be between 15 and 25%;

1285 5) Fresh cheeses (Petit Gervais type) appear to have a higher calcium absorption at around 42%.

1286 In the end, it remains difficult to draw firm conclusions as experimental designs and dairy
1287 products differ from one study to another. However, the fractional absorption of calcium measured
1288 in the rat is generally greater than that measured in humans. Moreover, according to the matrices, it
1289 is probably with the cheeses that the variations of the absorption of the calcium are the strongest,
1290 especially in the rat (Table 2). This is not surprising given the multiplicity of matrices and
1291 nutritional compositions in the large « family » of cheeses. Finally, Dr. Guéguen recalls that "The
1292 absorbability of calcium depends relatively little on its solubility in water, but especially on its
1293 solubility in acid medium (Heaney et al., 1990). If the absorption of calcium from milk is often
1294 taken as a reference, it must be admitted that it is not significantly higher than that of most calcium
1295 mineral salts (carbonate, phosphate, sulfate, chloride, etc.) and is sometimes a little lower than that
1296 of organic salts (lactate, gluconate, citrate, citromalate, etc.). However, differences between good
1297 sources of calcium remain low (Gueguen and Pointillart, 2000), the actual absorption coefficient (in
1298 humans) being most often found, under the most favorable physiological conditions, for milk as for
1299 good calcium salts, between 25 and 30% » (page S9) (Gueguen, 2005).

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1302 **General conclusions and perspectives**

1303 Depending on the nature of their matrix, milks, yogurts, fermented milks and cheeses have different
1304 effects on the bio-accessibility and bioavailability of their nutrients (Table 1 and Figure 1).

1305 Milks have a high protein digestibility of more than 95%. The structure of proteins (casein
1306 *versus* whey) and the physico-chemical characteristics of lipid droplets play a predominant role in
1307 the kinetics of bioavailability of amino acids and fatty acids, respectively. In particular, the
1308 viscosity, the type of emulsion, the size of the particles and possibly also the protein content,
1309 influence the digestion and absorption of the milk fat, and probably affects the lipemic responses.
1310 Concerning calcium, absorption in humans is around 20-30%. As with milks, yogurts have a high

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2 1311 digestibility of their protein fraction ($\geq 95\%$). For the lipid fraction, the data are too still few to
3 1312 conclude. Concerning calcium, its absorption is around 15-25% in humans. Fermentation appears to
4 1313 increase calcium solubilization during digestion. Since cheeses display a wide variety of matrices
5 1314 and nutritional composition, it is difficult to propose solid conclusions for the parameters studied in
6 1315 this report. However, some data can be synthesized: 1) the digestibility of cheese proteins is very
7 1316 high, as for milk and yogurts, *i.e.*, greater than 95%; 2) the kinetics of digestibility of the lipids
8 1317 depend in particular on the ratio of proteins/lipids and the texture of the matrix; 3) in human, the
9 1318 absorption of calcium is probably between 20 and 40% depending on the cheese matrices
10 1319 considered.

11 1320 The "matrix" effect of dairy products on the bioavailability of protein, lipid and calcium
12 1321 fractions remains a topic to be explored. In all cases, data collected up to now seem to show
13 1322 different behavior of kinetics of bioavailability of amino acids, fatty acids and calcium according to
14 1323 the physicochemical parameters of these matrices, including compactness, hardness, elasticity,
15 1324 protein/lipid ratio, P/Ca ratio, effect of ferments, size of fat globules, and possibly other qualitative
16 1325 parameters yet to be discovered. This could be of great interest for the development of innovative
17 1326 dairy products for older populations, sometimes in protein denutrition or with poor dentition,
18 1327 involving the development of dairy matrices with optimized metabolic effects by playing on gastric
19 1328 retention time and thus on the kinetics of release of the amino acids within bloodstream.

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22 1331 **Conflict of Interest**

23 1332 None: no funding was received for writing this review.

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26 1335 **Abbreviations**

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30 1339 **References**

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1695 **Figure legends**

1696 **Figure 1.** A synthetic view of the matrix effect of main dairy products on metabolim

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Table 1. Influence of main dairy matrices on the digestibility, bioaccessibility and/or bioavailability of proteins, lipids and calcium, and satiety^a

	Milks	Yogurts and fermented milks	Cheeses
Proteins	<p><i>In vitro</i> :</p> <ul style="list-style-type: none"> - After oral and gastric digestion, all proteins except β-lactoglobulin were degraded, with peptides being further degraded at the end of intestinal digestion - Concerning the β-lactoglobulin fatty acids and triglycerides would link to it preventing proteases access, and therefore requesting bile for further digestion - Skimmed cow milk > skimmed caprine goat milk - Heating milk has important impact on the hydrolysis kinetics of its protein fraction <p><i>In vivo</i> :</p> <ul style="list-style-type: none"> - Digestibility \geq 95% - The rate of digestion influences postprandial protein retention: casein (slow protein) versus whey (fast protein) - In the elderly, a fast protein results in better postprandial protein gain than a slow protein, contrary to what is observed in the young subject 	<p><i>In vitro</i> :</p> <ul style="list-style-type: none"> - Digestion of yoghurt results in different peptide profiles than milk <p><i>In vivo</i> :</p> <ul style="list-style-type: none"> - Digestibility \geq 95% - Proteolysis during fermentation may lead to the formation of new peptides during gastrointestinal digestion 	<p><i>In vitro</i> :</p> <ul style="list-style-type: none"> - More cohesive, compact, elastic and structured cheeses would disintegrate less rapidly during digestion, especially at the gastric level, resulting in slower proteolysis <p><i>In vivo</i> :</p> <ul style="list-style-type: none"> - Digestibility \geq 95%
Lipids	<p><i>In vitro</i> :</p> <ul style="list-style-type: none"> - The digested milk contains nearly 41% free fatty acids with respect to the total milkfat (this value corresponded to 100% of the triglycerides) ; - More short chain fatty acids are released after digestion compared to the relative distribution of total fatty acids in milk before digestion ; - The size of the lipid droplets influences the kinetics of lipolysis ; - The initial size of fat globules controls the type of free fatty acids generated but not the degree of 	<p><i>In vivo</i> :</p> <ul style="list-style-type: none"> - Fermented milk results in a significantly slower rate of gastric emptying, probably because of higher viscosity ; - Fermented milk results in a greater increase and a more rapid decrease in the content of triglycerides in all lipoprotein fractions. 	<p><i>In vitro</i> :</p> <ul style="list-style-type: none"> - The properties of the cheese matrix influence the access to the lipids of the cheese by controlling the release of the free fat and also via the restriction of the diffusion of the enzyme and the fatty acids ; - Calcium influences both the structure of the cheese and the kinetics of lipolysis. <p><i>In vivo</i> :</p> <ul style="list-style-type: none"> - Hard cheeses cause levels of LDL cholesterol, and probably total cholesterol, significantly lower than butter ;

	<p>lipolysis ;</p> <p>- The composition of the milk fat cell membrane affects the rate of lipid hydrolysis.</p> <p><i>In vivo :</i></p> <p>- Short- and medium-chain fatty acids are released faster than long-chain fatty acids ;</p> <p>- The viscosity, the type of emulsion, the size of the particles and possibly also the protein content, influence the digestion and absorption of dairy fat, and probably affects the lipemic response ;</p> <p>- The small fat globules release probably less atherogenic fatty acids.</p>	<p>- Two different cheeses can have a different impact on total cholesterol.</p>
Calcium	<p><i>In vitro :</i></p> <p>- Calcium is apparently almost completely solubilized, and fermentation of milk could further increase this solubilization ;</p> <p>- The dialysability of calcium varies from 20 to 28% without apparent effect of the fat content ;</p> <p>- In the presence of cereal products, solubilization of calcium is reduced.</p> <p><i>In vivo : rats</i></p> <p>- The apparent absorption of calcium varies from 74 to 80% ;</p> <p>- The absorption of calcium is decreased with aging.</p> <p><i>In vivo : humans</i></p> <p>- The absorption of calcium is between 17 and 55%, with a majority of studies giving values between 20 and 30% ;</p> <p>- The fractional absorption of milk calcium is similar to that of mineral water.</p>	<p><i>In vitro :</i></p> <p>- The dialysability of calcium varies from 28 to 33% without effect of the fat content or the acidity of the products ;</p> <p>- While the calcium fraction released is about 67%, this fraction is always lower in the presence of cereal products or additives.</p> <p><i>In vivo : rats</i></p> <p>- The apparent absorption of calcium varies from 72 to 83% ;</p> <p>- The apparent absorption of calcium from fermented milks is higher than that of normal yoghurt and that of milk.</p> <p><i>In vivo : humans</i></p> <p>- The fractional absorption of calcium seems to be between 15 and 25%.</p> <p><i>In vitro :</i></p> <p>- The calcium dialysability of cheese ranges from 14 to 32%, and may depend on the ratio of phosphorus to calcium (P/Ca).</p> <p><i>In vivo : rats</i></p> <p>- The apparent absorption of calcium varies from 12 to 90% according to cheese matrices ;</p> <p>- Absorption and retention of calcium is superior for fresh cheese compared to ripened or unripened cheese;</p> <p>- Calcium absorption of Cheddar cheese is decreased with aging;</p> <p>- Lipid content may increase calcium absorption and decrease bone resorption;</p> <p>- The refining time of a cheddar cheese has no significant effect on the absorption of calcium;</p> <p>- The absorbability of calcium is not increased by the addition of lactose (considered favorable to absorption and absent or in small quantity in most cheeses)</p> <p><i>In vivo : humans</i></p> <p>- There is a high probability that the bioavailability of calcium in cheese will not be different from that of calcium in milk;</p> <p>- Cheddar cheese has a fractional absorption of calcium (37%) slightly higher than milk (33%), processed cheese (33%) and yogurt (24%);</p>

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- Fresh cheeses appear to have a higher calcium absorption at about 42%;
 - The calcium absorption of a cooked pressed cheese is significantly higher than that of calcium from various plants.
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⁸1698 ⁹ *Syntheses and conclusions based on data from the literature presented in this report



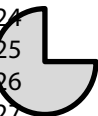
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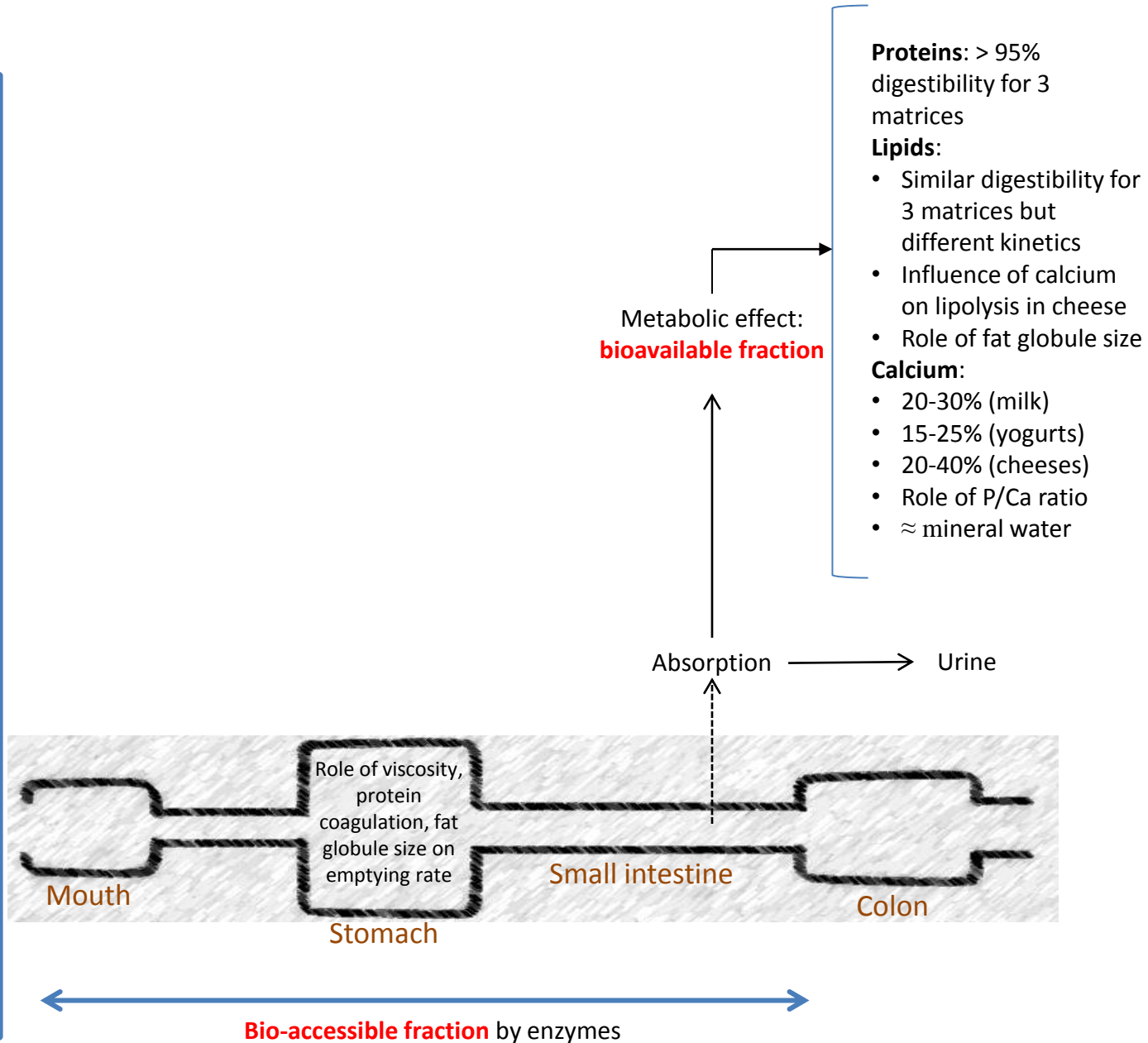
Tableau 2. Calcium apparent absorption from different dairy products as measured in adult rats*

Dairy products	Apparent absorption (%)
Whole cow milk	76, 74-77, 77
Skimmed milk	15, 69 ^a , 80
Plain yogurt	15, 72 ^a , 78
Probiotic yogurt	82-83
Fresh cheese (acid coagulation)	90
Ripened cheese	60-76
Unripened Ricotta cheese	57-61
White cheese	90
Monterey Jack cheese	53
Cheddar (31% fat)	20
Cheddar (7% fat)	12
Cheddar (35% fat, 0 day ripening)	78
Cheddar (35% fat, 70 days ripening)	73
L'Envol cheese (4% fat)	35
Goat fresh cheese curds	59 ^a

* Percentages separated by a comma correspond to separate studies. ^aGoat milk

Figure 1.

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- 2 
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- 5 **Milks: liquid matrices**
- 6
- 7 • Whey: rapid protein
- 8 • Casein: slow protein
- 9 • Small fat globule → less
- 10 atherogenic?
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- 12 • Short/medium-chain released
- 13 faster than long-chain fatty acids
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- 19 **Yogurts: semi-solid & viscous**
- 20 **matrices**
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- 22 • New peptides during fermentation
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- 28 **Cheeses: solid & semi-solid matrices**
- 29 • More cohesive/compact/elastic
- 30 → slower proteolysis
- 31 • ↑ Protein/lipid ratio → ↓
- 32 proteolysis & lipolysis (denser
- 33 protein network)
- 34
- 35 • Different effect on
- 36 cholesterolemia according to
- 37 matrices
- 38



- Proteins:** > 95% digestibility for 3 matrices
- Lipids:**
 - Similar digestibility for 3 matrices but different kinetics
 - Influence of calcium on lipolysis in cheese
 - Role of fat globule size
- Calcium:**
 - 20-30% (milk)
 - 15-25% (yogurts)
 - 20-40% (cheeses)
 - Role of P/Ca ratio
 - ≈ mineral water