Asensio-Grau, A.; Peinado, I.; Heredia Gutiérrez, AB.; Andrés Grau, AM. (2019). In vitro study of cheese digestion: Effect of type of cheese and intestinal conditions on macronutrients digestibility. LWT - Food Science and Technology. 113:1-7. https://doi.org/10.1016/j.lwt.2019.108278

In vitro study of cheese digestion: effect of type of cheese and intestinal conditions on macronutrients digestibility

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Abstract

Exocrine Pancreatic Insufficiency (EPI) implies maldigestion, being pancreatic enzyme replacement therapy the treatment to enhance digestibility. This study aims at analysing the influence of cheese-related factors and intestinal conditions on macronutrients digestibility. Fresh-cow, fresh-goat, mild and aged cheeses were in vitro digested under different intestinal conditions of pH (6 or 7), bile concentration (1 or 10mmol/L) and pancreatic enzymes (0-4000 LU/g fat) in order to in vitro mimic the intestinal conditions of a healthy adult and of an individual suffering of EPI. Under intestinal conditions of EPI (pH 6, bile 1mmol/L), lipids of fresh-goat and aged cheeses were more easily digested than those of fresh-cow and mild cheeses. In fact, 2000 LU/g fat of enzymatic dosage was enough to achieve a lipolysis extent of 80 and ~~100%~~ 100 % in aged and fresh-goat cheeses, respectively. In contrast, proteolysis was higher in fresh-cow cheese and ripened (mild or aged) than in fresh-goat one regardless the intestinal conditions. Only in ripened-cheeses, proteolysis significantly increased at dose of enzymes does.

Keywords: pancreatic insufficiency; cheese; lipolysis; proteolysis; fatty acids profile

1. Introduction

Food lipids are important in diets of infants and children, especially when digestive disorders occur such as exocrine pancreatic insufficiency (EPI), as they supply metabolic energy and phospholipids, the main constituents of biological membranes (Briefel, Reidy, Karwe, & Devaney, 2004). Among lipid-containing foods, dairy products are highly consumed by childhood population (Calvo-Lerma et al., 2019). Cheese is particularly rich in lipid and a good source of essential nutrients such as protein, bioactive peptides, vitamins and minerals (Walther, Schmid, Sieber, & Wehrmüller, 2008). The three major constituents of cheese are protein, fat and water, and all them conform their matrix structure. Protein matrix consists of casein particles that are bonded with calcium ions through electrostatic forces or hydrophobic aggregations, which entrap fat globules. Water content depends on the manufacturing process that directly influences lipid and protein content. Cheese can be consumed directly after its elaboration (fresh cheese), or after a ripening stage (ripened cheese). During ripening, proteolysis disaggregates the casein network that conforms the cheese matrix, while lipolysis is the main process determining the flavour. These physicochemical events might influence lipid and protein bioaccessibility (Ayala-Bribiesca, Lussier, Chabot, Turgeon, & Britten, 2016).Besides cheese processing, milk origin also determines protein and lipid content. 98% of dairy lipids are triacyglycerides (TAG) (Ayala-Bribiesca, Turgeon, & Britten, 2017); being the three predominant free fatty acids (FFA) forming part of TAG the following: palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$) and oleic acid ($C_{18:1}$ cis (n-9)) (Ceballos et al., 2009). The origin of milk (cow, goat or sheep milk), also influences lipid digestibility, resulting goat milk fat in better digestibility as compared to cow milk fat (Alférez et al., 2001).

The intestinal environment as well as the intrinsic matrix composition of food have been reported to influence the digestion process (Calvo-Lerma, Fornés-Ferrer, Heredia & Andrés, 2018; Hur, Lim, Decker, & McClements, 2011). Lipid digestion requires complex mechanisms, such as biliar secretion to emulsify fat globules (fat micelles), making them accessible for the pancreatic enzymes. However, gastrointestinal environment can vary among different individuals (Shani-Levi et al., 2017). Concretely,

in the EPI scenario, the obstruction of the pancreatic duct results in deficient secretion of pancreatic juice containing pancreatin and sodium bicarbonate. Besides, alteration of the biliary duct can lead to ~~a~~ reduced secretion of bile salts. This situation causes mal-digestion and mal-absorption, mainly of fats (Whitcomb et al., 2010).To revert the situation, oral pancreatic enzyme replacement therapy is the life-long treatment patients have to adhere to Turck et al., 2016.

In this context, the aim of the present study was to evaluate, by means of a static *in vitro* gastrointestinal digestion model, the influence of some intestinal conditions of pH (6 and7), bile concentration (1 and 10 mmol/L) and pancreatic supplementation (0-4000 Lipase Units (LU)/g fat) associated to EPI, on protein and lipid digestion in cheeses with different milk origin (cow or goat) and ripening time (mild or aged).

2. Materials and methods

2.1 Materials

Four types of cheese were used in this study. On the one hand, two cheeses of different ripening conditions but with the same milk composition (55 % cow, 25 % sheep and 20 % goat):mild-cheese (30 days of aging time)and aged-cheese (240 days of aging time). On the other hand, cheeses with the same elaboration process but different milk origin (100 % goat or 100 % cow): fresh goat cheese and fresh cow cheese, were also assessed. All cheeses were produced by "Queserías Entrepinares, S.A.U." and distributed in a local supermarket in Valencia (Spain).

The simulated digestive fluids were prepared with KCl, KH_2PO_4, NaCl, $NaHCO_3$, $MgCl_2$ $(H_2O)_6$, $(NH_4)_2CO_3$, $CaCl_2$, human α – amylase (1000 – 3000 U/ mg protein), pepsin from porcine gastric mucosa (≥2500 U/mg protein) and bovine bile extract all of them from Sigma-Aldrich Chemical Company (St Louis, MO, USA). The pancreatic enzymes supplements came from Kreon 10,000 LU (Mylan, USA), each capsule containing 150 mg of gastro-resistant microspheres including porcine pancreatic enzyme equivalent to 10,000 lipase units, 8,000 amylase units, and 600 protease units.

For the analytical determinations, Triton-X 100 %, trichloroacetic acid (TCA), hexane,and the analytical standards were acquired from Sigma-Aldrich Chemical Company (St Louis, MO, USA), and ethanol (95 % v/v for analysis), NaOH and HCl, were from AppliChem Panreac.

2.2 Experimental design

The experimental design consisted in two sets of experiments. In the first set, the dose of enzyme supplement was remained fixed at 2000 LU/g of fat, and the study variables were different combinations of intestinal pH and bile salts concentration (mmol/L): 6-1, 6-10, 7-1 and 7-10, with the purpose of analysing the impact of intestinal conditions on lipolysis and proteolysis. The intestinal condition of pH 6 and 1 mmol/L would represent the worst unfavourable intestinal scenario in EPI (Gelfond, Ma, Semler, & Borowitz, 2013; Seksik et al., 2018). The intestinal condition of pH 7 and 10 mmol/L bile salts would correspond to the gastrointestinal scenario of a healthy subject (Minekus et al., 2014). In the second set, the intestinal conditions of EPI were simulated (pH 6 – 1 (mmol/L)) and different doses of enzyme supplement (0, 1000, 2000, 3000 and 4000 LU/g of lipid) were tested, in order to assess the influence of supplement concentration on lipolysis and proteolysis. A blank of digested fluids in each intestinal condition was also analysed in the absence of food. All the experiments were performed at least in triplicate.

2.3 In vitro digestion

Fat, water, carbohydrate and protein contents in all cheeses were determined before digestion with the official methods (AOAC, 2000). In vitro digestion was conducted on the basis of the INFOGEST Cost Action international protocol (Minekus et al., 2014) with some modifications in order to simulate EPI conditions (Asensio-Grau, Peinado, Heredia, & Andrés, 2018). Digestion fluids were prepared from the corresponding stock solutions according to Minekus et al. (2014). Before each experiment, the enzymatic

activity was cheeked according to the protocol published by Carriere et al. (2000). The *In vitro* digestion process was conducted as follows:

Oral stage: Simulated Salivary Fluid (SSF; pH 7) was added to the cheese sample in a ratio 1:1 (w/v) properly homogenized using a kitchen blender for 3 min at 37 ºC (Vario Mixer, Ufesa 600 W). Human α – amylase was added as a part of SSF to reach a concentration in the salival mixture of 75 U/ml.

Gastric stage: Subsequently, Simulated Gastric Fluid (SGF; pH 3) was added to each tube in a 1:1 (v/v) ratio including pepsin to reach a concentration in the gastric mixture of 2000 U/ml. The pH of the mixtures was adjusted with HCl (1 N) to pH 3. Tubes were head-over-heels rotated at 55 rpm for 2 h at 37 ºC using Intell – Mixer RM – 2 (Elmi Ltd, Riga, LV – 1006, Latvia) in an incubator chamber (JP Selecta SA, Barcelona). The pancreatic supplement was added at the gastric stage, mimicking the incorporation of pancreatic enzyme replacement therapy to the digestion process.

Intestinal stage: After the gastric stage, Simulated Intestinal Fluid (SIF; pH 6 or 7) was added in 1:1 (v/v) ratio to each tube containing the gastric chime. The mixtures were adjusted to pH 6 or 7, depending on the experimental set with NaOH (1N). Then, samples were rotated head-over-heels at 55 rpm for 2 h at 37 ºC. During digestion, pH was controlled to keep the experimental conditions, as pH below 5.7 might inactivate lipase activity (González-Bacerio, Hernández, & Martínez, 2010; Prazeres, Garcia, & Cabral, 1994).

2.4 Analytical determinations

2.4.1 Matrix Degradation Index (MDI (%))

Matrix degradation Index (%) was calculated from the proportion of solids that were finely dispersed in the digested juice after the intestinal stage (Lamothe, Corbeil, Turgeon, & Britten, 2012).The total content of the tubes was centrifuged (4000 x g-force 20 min, 4 ºC) and then filtered on a metallic sieve (1.6 mm x 1.6 mm mesh)in order to separate the solid fraction. The liquid fraction was kept for lipolysis extent determination. The remaining liquid phase was freeze-dried (-40 °C and 1.25 mbar,

Telstar, Terrasa, Spain) and used for fatty acids profile analysis by gas chromatography. The solid large particles from digestion were transferred to an aluminium plate and then placed in a force air oven at 60 °C for 48 hours to determine the mass of large cheese particles.

2.4.2 Proteolysis

Proteolysis was determined by measuring the soluble protein fraction in trichloroacetic acid (TCA) (Lamothe, Azimy, & Bazinet, 2014) at different times (0, 10, 20, 60, 90 and 120 min) during gastric and intestinal stages. Aliquots of digested samples were extracted and TCA was added to a final concentration of 15 % (w/w), and then centrifuged at 4000 g-force for 15 min at 4 ºC. Then, the supernatant containing the hydrolysed peptides was mixed with glycine buffer, and the absorbance (OD) measured at 280 nm using a spectrophotometer (UV/vis, Beckman Coulter). Proteolysis was estimated by considering two parameters OD_{max} and $\Delta OD/h_{initial}$ from the mathematical model published by Bax, Aubry, Ferreira, Daudin, & Gatellier (2012) and using a Solver of Microsoft® Excel in order to estimate calculate-both parameters.

2.4.3 Lipolysis extent (%)

Aliquots from the liquid fraction of digested samples were diluted with a solution (5.6 % Triton X-100 and 6 % ethanol in water) to solubilize free fatty acids ensuring lipase activity inactivation (Lamothe, Corbeil, Turgeon, & Britten, 2012). FrFA release after digestion was measured by means of an enzymatic kit (Roche Diagnostics, Indianapolis, IN, USA) using a spectrophotometer (UV/vis, Beckman Coulter). Palmitic acid standard was used for quantitative determination of FFA. Lipolysis extent (%) was expressed as the percentage of total fatty acids released after complete digestion, considering the maximum release of 2 fatty acids per 1 molecule of triacylglycerol and the average molecular weight of milk triglycerides 741 g/mol (Hunter, 2001). Lipolysis of the studied cheeses was also determined before digestion, to estimate lipid hydrolysis during ripening.

144 *2.4.4. Free fatty acid profile*

145 Chromatography mass spectrometry (GC-MS) was used for identification of FFA from cheese
146 before and after digestion. Undigested samples were subjected to a Soxhlet extraction (AOAC, 2000),
147 while digested samples were extracted with hexane. Lipid samples needed a transesterification from fatty
148 acids to methyl esters (FAMEs) with BF_3 and methanol at 20 ºC ~~20ºC~~ according to the IUPAC standard
149 method (Yaich et al., 2011). Then, samples were analysed with an Agilent 5977A system and an HP-5MS
150 UI (Agilent) (Colum: 30 m x 0.25 mm, 0.25 μm film thickness) with helium as carrier agent (1 ml/min).
151 Extraction, esterification and the analysis conditions were previously described by Paz-Yépez, Peinado,
152 Heredia, & Andrés (2018).

153 ***2.5 Statistical analyses***

154 Simple ANOVA analyses were performed to assess the statistical significance of the intestinal
155 conditions variables, milk origin and maturation stage on MDI, proteolysis, lipolysis extent, and free fatty
156 acid profile in digested cheeses. Statgraphics Centurion was used and the analyses were conducted with at
157 least a significance of 95 % (*p-value* < *0.05*).

158

159 **3. Results and discussion**

160 ***3.1 Influence of the intestinal pH and bile concentration on macronutrients digestibility of different***
161 ***fresh and ripened cheeses***

162 As previously mentioned, the studied cheeses were characterized before digestion in terms of fat,
163 protein and carbohydrate contents (g of each macronutrient/ g of dry matter). Fresh-cow cheese
164 (0.32±0.03) presented lower fat content than the fresh-goat (0.50±0.06), mild (0.544±0.003) and aged
165 cheeses (0.51±0.04). However, the protein content was similar in all of them (≈0.3 g/g dry matter).
166 Regarding carbohydrate content, as expected, ripened cheeses presented less content (aged cheese
167 (0.015±0.002) and mild cheese (0.022±0.003)) compared to fresh ones (fresh cow cheese (0.18±0.05) and
168 fresh goat cheese (0.097±0.04)).These differences can be attributed to the different composition of cow

and goat milks, in terms of lipids for instance, and to the cheese-making process. In case of fresh cheeses, the rennet is immediately added after milk pasteurization, when temperature reaches 32-37 ºC ~~32-37ºC~~, and then mixed to coagulate the milk. After 30 minutes, draining and pressing proceed. Conversely, ripened cheeses are kept on a maturating chamber after coagulation, where the temperature and time of the process are controlled. This situation leads to ripened cheeses resulting in an additional loss of moisture content with an increase of protein and lipids in ripened cheeses compared to fresh ones, but in a lower carbohydrate content due to bacterial conversion of carbohydrates into other metabolic compounds.

Table 1 reports the values for MDI (%) and lipolysis extent (%) achieved in cheeses digested at a fixed enzyme dose of 2000 LU/ g fat but different gastrointestinal conditions of pH (6 or 7) and bile concentration (1 or 10 mmol/L). As observed, both fresh cheeses presented higher MDI (%) under healthy conditions of pH 7 and bile concentration of 10 mmol/L; whereas in ripened cheeses, similar values of MDI (%) (between 73 and 81) were found regardless the intestinal conditions, and being higher than the values obtained for fresh varieties. During fresh cheese production, and directly after acidic or enzymatic coagulation of caseins, cheeses are pressed and packaged resulting in softer structures than those of the ripened ones, but with a very stable three-dimensional casein matrix (Pastorino, Hansen, & McMahon, 2003). During the further ripening stage applied in the production of aged cheeses, proteolysis, lipolysis and the metabolism of residual lactose, lactate and citrate, are the three primary routes by which biochemical activity continues. The relative importance of each of these processes is largely dependent on cheese variety; however, proteolysis has been pointed out as the most complex mechanism, therefore playing a significant role during ripening of nearly all varieties. In fact, Karaman and Akalin (2013) reported a decrease of the hardness and cohesiveness in ripened cheeses as a consequence of proteolysis. In the same way, lipolysis contributes to the volatile compounds profile as well as to unctuousness. Therefore, proteolysis and lipolysis occurring during ripening stage could favour further matrix degradation during the gastrointestinal track.

Concerning lipolysis extent after digestion, complete lipolysis (≈ 100 %) was achieved in most of the studied cheeses under the standard healthy intestinal conditions (pH 7 and bile concentration 10 mmol/L). However, these results also show that the different manufacturing processes can influence the bioavailability of lipids at suboptimal intestinal conditions of 6 and bile concentration of 1 mmol/L. Concretely, lipolysis was found to be higher in aged cheese and fresh goat cheese than in mild and fresh cow cheese at these intestinal conditions. Aged cheese showed a significant higher lipolysis extent compared to mild cheese, being the only difference between them the aging time: 30 days for mild and 240 days for aged cheese. The lipolysis extent found in these cheeses before digestion was 1.6±0.3 % and 3.4±0.5 %, respectively; while the extent of lipolysis in fresh cow and fresh goat cheese was around 0.4±0.2 % in both cases. During ripening process, products of hydrolysis could enhance the further lipolysis during digestion because of their emulsifying capacity (Maldonado-Valderrama, Wilde, MacIerzanka & MacKie, 2011)

Bile concentration played a crucial significant role on lipolysis attained in fresh cow cheese at both pH 6 and 7, and aged and mild cheese at pH 7. However, lipolysis extent was not affected in fresh goat cheese, with complete lipid hydrolysis regardless the intestinal pH or bile salt concentration. Goat milk is richer in short chain fatty acids than cow milk. Short chain fatty acids are easier to hydrolyse by lipases, becoming the role of bile salts or pH less important (Arora, Bhojak & Joshi, 2013). Therefore, these results revealed that triglycerides from fresh goat cheese are more digestible than in fresh cow type, which increases their nutritional value and their health benefits.

FFA profiles resulting from cheese digestion were determined in two intestinal scenarios, corresponding to the healthy situation (pH 7 and bile 10 mmol/L) and the EPI conditions (pH 6 and bile 1 mmol/L) in order to deeper understand the consequences of the EPI disorder on lipid mal-digestion.

Additionally, FFA profile of mild, aged, fresh goat and fresh cow cheeses (g FFA/100 g total fatty acids) was analysed prior digestion, and results are gathered in **Table 2.**.The predominant FFAs found in all cheeses were lauric acid ($C_{12:0}$), palmitic acid ($C_{16:0}$)and stearic acid ($C_{18:0}$) above saturated FFA; and

218 oleic acid ($C_{18:1}$) over the unsaturated ones. Concretely, these four FFA entail the 85.8, 86.4, 83.8 and
219 83.7 % of total FFA in aged, mild, fresh goat and fresh cow cheeses, respectively. Particularly, the main
220 differences between fresh goat and fresh cow cheeses were found in the amounts of saturated fatty acids
221 ($C_{6:0}$, $C_{8:0,}$ $C_{10:0}$, $C_{12:0}$ and $C_{20:0}$). Similar FFA distribution was reported for fresh goat and fresh cow
222 cheeses in other studies (Ceballos et al., 2009; Rodríguez-Alcalá, Harte & Fontecha, 2009), differences
223 beingattributed to milk origin. Aged and mild cheeses presented a very similar FFA profile, as expected,
224 since they are made from the same mixture of milks, and in the same proportion, being the only
225 difference between them the aging time. However, aged cheese presented a slightly, although statistically
226 significant, higher amount of $C_{12:0}$ (16.93 %) and $C_{14:0}$ (3.007 %); while mild cheese showed a higher
227 content in $C_{18:0}$ (17.58%). The difference found in the content of myristic acid ($C_{14:0}$) between fresh and
228 ripened cheeses might be attributed to the action of lipolytic agents during the maturation process, such as
229 enzymes from milk, rennet and the microflora.

230 Regarding the influence of intestinal conditions on the FFA profile after digestion (Figure 1), the
231 optimal conditions of pH 7 and bile 10 mmol/Lslightly increased the FFA released compared to the EPI
232 conditions. An exception of this tendency was the FFA found in the digested aged cheese, for which
233 slightly higher contents of saturated and unsaturated medium-long-chain fatty acids ($C_{16:0,}$ $C_{18:0}$, $C_{18:1\ c}$,
234 $C_{18:1\ t}$ and C_{20}) were found at pH 6 and 1 mmol/L of bile salts. When comparing FFA profile of the four
235 digested cheeses, one gram of digested fat of fresh goat cheese presented considerably higher quantities
236 of all FFA than the same fat amount of fresh cow cheese. Likewise, the FFA profile of digested aged
237 cheese was richer in all the identified FFA than mild cheese. These results again evidence the easier
238 digestibility of fresh cheeses made from goat milk and cheeses subjected to long maturation time.
239 Therefore, consumption of fresh goat and aged cheeses would be advisable for EPI patients with
240 suboptimal intestinal conditions.

241 Concerning proteolysis, **Table 3** shows the initial slope ($\Delta OD/h_{initial}$) and the maximum protein
242 hydrolysis (OD_{max}) of the different cheeses digested under different intestinal conditions. OD_{max} can be

243 considered as an indicator of the proteolysis extent, while the initial slope ($\Delta OD/h_{initial}$) refers to the
244 kinetics of the initial proteolytic reactions (Bax, Aubry, Ferreira, Daudin, & Gatellier, 2012). Both
245 parameters were determined during and at the end of gastric and intestinal stages. Remarkably, all the
246 studied cheeses presented similar protein content (g/g dry matter).

247 According to the obtained results, proteolysis resulting from gastric pepsin activity was higher in
248 aged and fresh cow cheeses, and much lower than in the intestinal stage for all cheeses. The hydrolysis of
249 protein achieved in fresh cow cheese is especially relevant because the Protease Units (PU)/g protein was
250 lower for this cheese in comparison with the others. Other studies, however, showed that goat milk
251 caseins were more efficiently digested compared to cow milk ones (Hodgkinson, Wallace, Boggs,
252 Broadhurst, & Prosser, 2018), in our study, gastric pepsin seemed to present more affinity for caseins of
253 cow cheese than for other kind of cheeses. Bovine milk proteins are similar to those of goat milk, but they
254 present different genetic polymorphisms. These differences are due to amino acids substitutions in protein
255 chains, and lead to different digestibility fates (Haenlein, 2004).

256 On the other hand, higher proteolysis (OD_{max}) was found at the end of gastrointestinal digestion
257 for both ripened cheeses when compared to fresh ones, and particularly than in fresh goat cheese that was
258 digested under similar PU/ g protein, and thus comparable to the ripened ones. As previously mentioned,
259 both, salting and ripening stages, favoured the proteolytic activity, giving as a result an increase of free
260 amino acids and small peptides in ripened cheese, coming from casein degradation (McSweeney, 2004).
261 As it was expected, pH 7 instead of 6 promoted higher proteolysis extent. In fact, it is well-known that the
262 optimal pancreatic proteases activity is around pH 7.5. However, neither intestinal pH nor bile
263 concentration seemed to determine kinetics ($\Delta OD/h_{initial}$) of the initial proteolytic reactions.

3.2 Influence of oral pancreatic supplementation on macronutrients digestibility of different fresh and ripened cheeses

The impact of the oral supplement dose on macronutrient digestibility was also assessed. **Figure 2** shows the results of the MDI (%) and lipolysis extent (%) at different enzymatic dosages (1000-4000 LU/g lipid) and at EPI fixed intestinal conditions of pH 6 and bile concentration 1 mmol/L. **Table 4** gathers the results of proteolysis under the same conditions. As previously stated ~~commented~~, MDI (%) did not seem to depend on intestinal conditions (**Table 1**), but on cheese-related factors and especially of ripening. Similarly, the supplementation with pancreatin only increased MDI (%) of digested fresh goat cheese, irrespective from the dosage. On the contrary, lipolysis extent (%) was highly dependent on pancreatin dose, increasing gradually as long as it did~~.~~ **Figure 2** shows that fresh goat cheese reached the maximum value of lipolysis extent (≈100 % ~~100%~~) at 2000 LU/ g fat. Fresh cow cheese, in contrast, required a higher dose (4000 LU/g fat) to reach a lipolysis extent of ≈65 % ~~65%~~. In fact, fresh cow cheese, 100 %made from cow milk, seemed to be the less digestible in terms of lipids. The difference might be due to the fat origin from milk (cow or goat). Goat milk presents higher concentration of short and medium chain fatty acids (C6 to C12) than cow milk. Consequently, this matrix allowed for greater release of free fatty acids. Some authors reported that goat milk has smaller fat globule size than cow milk, whereby led to greater rates of lipolysis in cheese made from goat milk(Ceballos et al., 2009; Logan et al., 2017; Park, 2001). Lower fat globule size leads to an increase of the total fat globules surface, apparently enhancing fat digestibility. Furthermore, aging time also affected lipolysis. The highest lipolysis extent in digested aged cheese (≈80%) and digested mild cheese (≈45%) was achieved at 2000 LU/ g fat. In both cheeses, the increment of pancreatic dose above 2000 LU/ g fat did not improve ~~the~~ lipid digestibility.

As shown in **Table 4,** the initial kinetics of proteolysis was faster (higher values of initial slope) in cheeses subjected to maturation than in fresh ones, independently of the enzymatic supplement dosage.

289 Small peptides and free amino acids resulting from proteolysis occurring during aging act as bio-
290 catalysers, enhancing the kinetics of the further proteolysis during digestion (Tavano, 2013). Proteolysis
291 occurred faster in fresh cow cheese than in fresh goat cheese, considering the results of initial slope at
292 2000 LU/ g lipid (194 PU/g protein) in fresh goat cheese, and 3000 LU/ g lipid (171 PU/g protein) in
293 fresh cow. Overall, **Table 4** illustrates that high doses of enzyme supplement seem to be associated with a
294 reduction of the initial slope in aged and fresh cow cheeses. These results can validate the hypothesis that
295 proteolysis extent decreases when a certain dose of enzyme supplement is present. This result could be
296 attributed to two phenomena. Enzyme auto-aggregation, over a certain limit of concentration, together
297 with product inhibition, could leads proteases inactivation. This second phenomenon could be especially
298 relevant in *in vitro* static models of digestion because of products of proteolysis are not gradually
299 removed from the system. In terms of maximum proteolysis, similar values for fresh cheeses at the same
300 PU/g protein were found. On the other hand, the presence of pancreatic proteases was essential to
301 accomplish casein hydrolysis during intestinal stage, but without significant relevance of the protease
302 dose. During cheese manufacturing, rennet is added to milk causing casein hydrolysis and leaving milk
303 serum free. As a result, an increase in the attractive forces is produced between micelles found in milk,
304 resulting in the formation of casein aggregates that maintain fat globules and serum retained inside the
305 protein matrix (McSweeney, 2004).The importance of protein hydrolysis and lipolysis during cheese
306 processing should be considered since the resultant matrices of milk and cheese are completely different.

307 **4. Conclusions**

308 The present study points out that the type of cheese and together with the host-individual
309 gastrointestinal conditions influence lipids and proteins digestibility. Even if lipolysis was completed
310 (≈100 %) for all cheeses under the healthy intestinal conditions; goat-fresh cheese and aged cheese
311 achieved higher lipolysis extent under EPI conditions lipolysis than cow-fresh cheese and mild-cheese.

312 Concerning protein digestibility, it was only dependent on the characteristics of cheeses, being
313 higher in fresh-cow cheeses and matured ones than in fresh-goat cheese. Results also demonstrate that

some dairy matrix properties, milk origin and ripening time, greatly affect lipolysis in the EPI situation. Concretely, aged and fresh-goat cheeses reached the maximum value of lipolysis extent, 80 % and 100 % respectively, at 2000 LU/g fat; whilst lipolysis remained incomplete in fresh-cow cheese and mild-cheeses even at the highest dose of 4000 LU/g fat.

To conclude, this study has unveiled the implication of food characteristics and host-related factors on lipid and protein digestibility in cheese products. Our findings could contribute to establish dietary recommendations for pancreatic insufficient patients, including the promotion of matured cheese consumption, as it would be the most easily digested.

5. Acknowledgements

Authors of this paper, on behalf of MyCyFAPP consortium, acknowledge the European Union and the Horizon 2020 Research and Innovation Framework Programme for funding the above-mentioned project under grant agreement number 643806. The authors would like to thank the Conselleria de Educació i Investigació de la Generalitat Valenciana for the PhD scholarship given to Andrea Asensio Grau.

6. References

AOAC (2000). Official methods of analysis of AOAC International. (17th ed.). Gaithersberg, Maryland: *Association of Official Chemists*.Agnihotri, M. K., & Prasad, S. (1993). Biochemistry and processing of goat milk and milk products. *Small Ruminant Research*, *12*, 151–170. https://doi.org/10.1016/0921-4488(93)90080-2

Alférez, M. J. M., Barrionuevo, M., López Aliaga, I., Sanz-Sampelayo, M. R., Lisbona, F., Robles, J. C., & Campos, M. S. (2001). Digestive utilization of goat and cow milk fat in malabsorption syndrome. *Journal of Dairy Research*, *68*(3), 451–461. https://doi.org/10.1017/S0022029901004903

Arora, R., Bhojak, N., & Joshi, R. (2013). Comparative aspacts of goat and cow milk. *Int. J. Engineering*

Sci. Invention, *2*(1), 7–10.

Asensio-Grau, A., Peinado, I., Heredia, A., & Andrés, A. (2018). Effect of cooking methods and intestinal conditions on lipolysis , proteolysis and xanthophylls bioaccessibility of eggs. *Journal of Functional Foods*, *46*(May), 579–586. https://doi.org/10.1016/j.jff.2018.05.025

Ayala-Bribiesca, E., Lussier, M., Chabot, D., Turgeon, S. L., & Britten, M. (2016). Effect of calcium enrichment of Cheddar cheese on its structure, in vitro digestion and lipid bioaccessibility. *International Dairy Journal*, *53*, 1–9. https://doi.org/10.1016/j.idairyj.2015.09.002

Ayala-Bribiesca, E., Turgeon, S. L., & Britten, M. (2017). Effect of calcium on fatty acid bioaccessibility during in vitro digestion of Cheddar-type cheeses prepared with different milk fat fractions. *Journal of Dairy Science*, *100*(4), 2454–2470. https://doi.org/10.3168/jds.2016-11902

Bax, M., Aubry, L., Ferreira, C., Daudin, J., & Gatellier, P. (2012). Cooking temperature is a key derminant of in vitro meat protein digestion rate: investigation of underlying mechanisms. https://doi.org/10.1021/jf205280y

Briefel, R. R., Reidy, K., Karwe, V., & Devaney, B. (2004). Feeding Infants and Toddlers Study: Improvements needed in meeting infant feeding recommendations. *Journal of the American Dietetic Association*, *104*(SUPPL. 1), 31–37. https://doi.org/10.1016/j.jada.2003.10.020

Calvo-Lerma, J., Fornés-Ferrer, V., Heredia, A., & Andrés, A. (2018). In vitro digestion of lipids in real foods: influence of lipid organization within the food matrix and interactions with nonlipid components. *Journal of Food Science*, *83*(10), 2629–2637. https://doi.org/10.1111/1750-3841.14343

Calvo-Lerma, J., Hulst, J., Boon, M., Martins, T., Ruperto, M., Colombo, C., … Ribes-Koninckx, C. (2019). The relative contribution of food groups to macronutrient intake in children with cystic fibrosis: a european multicenter assessment. *Journal of the Academy of Nutrition and Dietetics*. https://doi.org/10.1016/j.jand.2019.01.003

Carriere, F., Renou, C., Lopez, V., De Caro, J., Ferrato, F., Lengsfeld, H., … Verger, R. (2000). The specific activities of human digestive lipases measured from the in vivo and in vitro lipolysis of test

meals. *Gastroenterology*, *119*(4), 949–960. https://doi.org/10.1053/gast.2000.18140

Ceballos, L. S., Morales, E. R., de la Torre Adarve, G., Castro, J. D., Martínez, L. P., & Sampelayo, M. R. S. (2009). Composition of goat and cow milk produced under similar conditions and analyzed by identical methodology. *Journal of Food Composition and Analysis*, *22*(4), 322–329. https://doi.org/10.1016/j.jfca.2008.10.020

Gelfond, D., Ma, C., Semler, J., & Borowitz, D. (2013). Intestinal ph and gastrointestinal transit profiles in cystic fibrosis patients measured by wireless motility capsule. *Digestive Diseases and Sciences*, *58*(8), 2275–2281. https://doi.org/10.1007/s10620-012-2209-1

González-Bacerio, J., Hernández, J. R., & Martínez, A. D. M. (2010). Las lipasas: enzimas con potencial para el desarrollo de biocatalizadores inmovilizados por adsorción interfacial. *Revista Colombiana de Biotecnología*, *12*(1), 124–140. Retrieved from http://www.revistas.unal.edu.co/index.php/biotecnologia/article/view/15574/38075

Haenlein, G. F. W. (2004). Goat milk in human nutrition. *Small Ruminant Research*, *51*(2), 155–163. https://doi.org/10.1016/j.smallrumres.2003.08.010

Hodgkinson, A. J., Wallace, O. A. M., Boggs, I., Broadhurst, M., & Prosser, C. G. (2018). Gastric digestion of cow and goat milk: Impact of infant and young child in vitro digestion conditions. *Food Chemistry*, *245*(October 2017), 275–281. https://doi.org/10.1016/j.foodchem.2017.10.028

Hunter, J. E. (2001). Studies on Effects of Dietary Fatty Acids as Related to Their Position on Triglycerides, *36*(7).

Hur, S. J., Lim, B. O., Decker, E. A., & McClements, D. J. (2011). In vitro human digestion models for food applications. *Food Chemistry*, *125*(1), 1–12. https://doi.org/10.1016/j.foodchem.2010.08.036

Karaman, A. D., & Akalin, A. S. (2013). Improving quality characteristics of reduced and low fat Turkish white cheeses using homogenized cream. *LWT - Food Science and Technology*, *50*(2), 503–510. https://doi.org/10.1016/j.lwt.2012.08.017

Lamothe, S., Azimy, N., & Bazinet, L. (2014). Function Interaction of green tea polyphenols with dairy

matrices in a simulated gastrointestinal environment. *Food & Function*, *5*, 2621–2631. https://doi.org/10.1039/C4FO00203B

Lamothe, S., Corbeil, M.-M., Turgeon, S. L., & Britten, M. (2012). Influence of cheese matrix on lipid digestion in a simulated gastro-intestinal environment. *Food & Function*, *3*(7), 724. https://doi.org/10.1039/c2fo10256k

Logan, A., Xu, M., Day, L., Singh, T., Moore, S. C., Mazzonetto, M., & Augustin, M. A. (2017). Milk fat globule size affects Cheddar cheese properties. *International Dairy Journal*, *70*, 46–54. https://doi.org/10.1016/j.idairyj.2016.11.003

Maldonado-Valderrama, J., Wilde, P., MacIerzanka, A., & MacKie, A. (2011). The role of bile salts in digestion. *Advances in Colloid and Interface Science*, *165*(1), 36–46. https://doi.org/10.1016/j.cis.2010.12.002

McSweeney, P. L. H. (2004). Biochemistry of cheese ripening: Introduction and overview. *Cheese: Chemistry, Physics and Microbiology*, *1*(C), 347–360. https://doi.org/10.1016/S1874-558X(04)80073-3

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., … Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food – an international consensus. *Food Funct. Food Funct*, *5*(5), 1113–1124. https://doi.org/10.1039/c3fo60702j

Park, Y. W. (2001). Proteolysis and lipolysis of Goat's milk cheese. *Journal of Dairy Science*, *84*(E. Suppl.), E84–E92. https://doi.org/10.3168/jds.S0022-0302(01)70202-0

Pastorino, A. J., Hansen, C. L., & McMahon, D. J. (2003). Effect of salt on structure-function relationships of cheese. *Journal of Dairy Science*, *86*(1), 60–69. https://doi.org/10.3168/jds.S0022-0302(03)73584-X

Paz-Yépez, C., Peinado, I., Heredia, A., & Andrés, A. (2018). Influence of particle size and intestinal conditions on in vitro lipid and protein digestibility of walnuts and peanuts. *Food Research International*, *119*(June 2018), 951–959. https://doi.org/10.1016/j.foodres.2018.11.014

414 Prazeres, D. M. F., Garcia, F. A. P., & Cabral, M. S. (1994). Continuous lipolysis in a reversed micellar
415 membrane bioreactor. *Bioprocess Engineering-*, *10*(1), 21–27.
416 Rodríguez-Alcalá, L. M., Harte, F., & Fontecha, J. (2009). Fatty acid profile and CLA isomers content of
417 cow, ewe and goat milks processed by high pressure homogenization. *Innovative Food Science and*
418 *Emerging Technologies*, *10*(1), 32–36. https://doi.org/10.1016/j.ifset.2008.10.003
419 Seksik, P., Rainteau, D., Wolf, C., Humbert, L., Laugier, R., Tuvignon, N., & Carrière, F. (2018).
420 Postprandial bile acid levels in intestine and plasma reveal altered biliary circulation in chronic
421 pancreatitis patients. *Journal of Lipid Research*, *59*(11), 2202–2213.
422 https://doi.org/10.1194/jlr.m084830
423 Shani-Levi, C., Alvito, P., Andrés, A., Assunção, R., Barberá, R., Blanquet-Diot, S., … Lesmes, U.
424 (2017). Extending in vitro digestion models to specific human populations: Perspectives, practical
425 tools and bio-relevant information. *Trends in Food Science and Technology*, *60*, 52–63.
426 https://doi.org/10.1016/j.tifs.2016.10.017
427 Tavano, O. L. (2013). Protein hydrolysis using proteases: An important tool for food biotechnology.
428 *Journal of Molecular Catalysis B: Enzymatic*, *90*, 1–11.
429 https://doi.org/10.1016/j.molcatb.2013.01.011
430 Turck, D., Braegger, C. P., Colombo, C., Declercq, D., Morton, A., Pancheva, R., … Wilschanski, M.
431 (2016). ESPEN-ESPGHAN-ECFS guidelines on nutrition care for infants , children , and adults with
432 cystic fi brosis. *Clinical Nutrition*, *35*(3), 557–577. https://doi.org/10.1016/j.clnu.2016.03.004
433 Weaver, C. M., & Heaney, R. P. (2005). Modern Nutrition in Health and Disease. *Modern Nutrition in*
434 *Health and Disease*, (October).
435 Whitcomb, D. C., Lehman, G. A., Vasileva, G., Malecka-Panas, E., Gubergrits, N., Shen, Y., … Caras, S.
436 (2010). Pancrelipase delayed-release capsules (CREON) for exocrine pancreatic insufficiency due to
437 chronic pancreatitis or pancreatic surgery: A double-blind randomized trial. *American Journal of*
438 *Gastroenterology*, *105*(10), 2276–2286. https://doi.org/10.1038/ajg.2010.201

Yaich, H., Garna, H., Besbes, S., Paquot, M., Blecker, C., & Attia, H. (2011). Chemical composition and functional properties of *Ulva lactuca* seaweed collected in Tunisia. *Food Chemistry*, *128*(4), 895–901. https://doi.org/10.1016/j.foodchem.2011.03.114

Figure captions

Figure 1. FFA profile of different cheese matrices-after in vitro digestion simulating two intestinal scenarios corresponding to a healthy adult (pH 7 and bile 10 mmol/L) and the most disadvantageous EPI conditions (pH 6 and bile 1 mmol/L). A: pH 6 Bile 1 mmol/Lfresh goat cheese ; pH 6 Bile 1 mmol/Lfresh cow cheese ; pH 7 Bile 10 mmol/Lfresh goat cheese ; pH 7 Bile 10 mmol/Lfresh cow cheese . B: pH 6 Bile 1 mmol/Lmild cheese ; pH 6 Bile 1 mmol/Laged cheese ; pH 7 Bile 10 mmol/Lmild cheese ; pH 7 Bile 10 mmol/Laged cheese .

$^{a-c}$ Letters refer to the homogenous groups obtained for different cheese (mild, aged, fresh cow and fresh goat cheeses) for the same fatty acid (C6, C8, C10, C12, C14, C16, C18, C18:1c, C18:1t, C18:2 and C20) and at a statistical significance of statistical significance of 95 % (*p-value* < *0.05*).

Figure 2. Matrix degradation index (%) (A) and Lipolysis extent (%) (B) obtained for the different cheese matrices (mild cheese ; aged cheese ; fresh cow cheese ; fresh goat cheese)after in vitro digestion at fixed duodenal conditions of pH 6 and Bile concentration 1 mmol/L using different doses of Kreon (0-4000 LU/ g fat). $^{a-c}$ Letters refer to the homogenous groups obtained for different doses (0 – 4000) for the same cheese matrix (mild, aged, fresh cow and fresh goat cheeses) at a statistical significance of 95% (*p-value* < *0.05*).