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CIÊNCIA ANIMAL- ESPECIALIDADE NUTRIÇÃO

Impact of origin and production system on milk composition and consequent effects on human nutrition and health

Susana Marques Pinho

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D.ICBAS 2022

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Tese de Candidatura ao grau de Doutor em Ciência Animal, Especialidade em Nutrição;
Programa Doutoral da Universidade do Porto (Instituto de Ciências Biomédicas de Abel Salazar).

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Thesis for applying to a Doctor degree in Animal Sciences, Specialty in Nutrition;
Doctoral Programme of the University of Porto (School of Medicine and Biomedical Sciences).

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FUNDING

The present thesis was performed under the SANFEED Program (Sustainable Animal Nutrition and Feeding), a Doctoral Animal Science Programme in an industrial setting funded by the Portuguese Foundation for Science and Technology (FCT- Fundação para a Ciência e a Tecnologia) and coordinated by the School of Medicine and Biomedical Sciences (ICBAS) from the University of Porto (PDE/0023/2013). This thesis was developed under the partnership of Soja de Portugal – Sociedade Gestora de Participações Sociais, S.A. through its company Sorgal – Sociedade de Óleos e Rações, S.A. (supervision of Rui Mário Miranda Alves – commercial director, zootechnical engineer), Laboratório Associado para a Química Verde/Rede de Química e Tecnologia (LAQV/REQUIMTE), and Faculty of Pharmacy, University of Porto.

Susana C.M. Pinho received financial support of FCT - Fundação para a Ciência e a Tecnologia and Soja de Portugal S.A., through the grants PD/BDE/135539/2018 and COVID/BDE/152525/2022.

The experimental work conducted on this thesis was funded by FCT - Fundação para a Ciência e a Tecnologia in the framework of the project DIETxPOSOME (PTDC/SAU-NUT/6061/2020) and by UIDB/50006/2020.

DISSEMINATION

Publications in Internationals Peer-Reviewed Journals

Pinho, S. C. M., Faria, M. A., Melo, A., Pinto, E., Almeida, A., Alves, R., Cabrita, A. R. J., Fonseca, A. J. M., & Ferreira, I. M. P. L. V. O. (2021). Effect of skimmed milk on intestinal tract: Prevention of increased reactive oxygen species and nitric oxide formation. *International Dairy Journal,* 118(105046). https://doi.org/10.1016/j.idairyj.2021.105046.

Pinho, S. C. M., Faria, M., Casal, S., Sobral, M. M. C., Alves, R., Cabrita, A. R. J., Fonseca, A. J. M., & Ferreira, I. M. P. L. V. O. (2021). Explore gastric lipolysis and lipid oxidation of conventional versus pasture-based milk by a semi-dynamic *in vitro* digestion model. *Journal of Agricultural and Food Chemistry*, 69(47) 14241-14249. https://doi.org/10.1021/acs.jafc.1c03150.

Pinho, S. C. M., Faria, M. A., Alves, R., Cabrita, A. R. J., Fonseca, A. J. M., & Ferreira, I. M. P. L. V. O. Gastric epithelial response to milk fat using the semi-dynamic INFOGEST digestion model coupled with NCI-N87 cells. *Submitted*.

Oral communications

Pinho, S. C. M., Faria, M. A., Alves, R., Cabrita, A. R. J., Fonseca, A. J. M., & Ferreira, I. M. P. L. V. O. (28-29th April 2022). Whole milk effect in mRNA expression of gastric epithelium. *DIETimpact on health* – Faculty of Pharmacy of the University of Porto.

Pinho, S. C. M., Faria, M. A., Melo, A., Pinto, E., Almeida, A. Alves, R., Cabrita, A. R. J., Fonseca, A. J. M., & Ferreira, I. M. P. L. V. O. (29-30th October 2021). Potential of milk consumption in the protection of intestinal tract. *XXII National Congress of Portuguese Zootechnics* – Online.

Pinho, S. C. M., Faria, M., Casal, S., Sobral, M. M. C., Alves, R., Cabrita, A. R. J., Fonseca, A. J. M., & Ferreira I. M. P. L. V. O. (17th September 2021). Conventional versus pasture-based cow's milk: simulated human gastric lipolysis and lipid oxidation. *VIII Annual Workshop for the Doctoral Program in Animal Science* – ICBAS, School of Medicine and Biomedical Science of the University of Porto.

Pinho, S. C. M., Faria, M. A., Melo, A., Pinto, E., Almeida, A., Alves, R., Cabrita, A. R. J., Fonseca, A. J. M., & Ferreira, I. M. P. L. V. O (20th-21st May 2021). Potential of milk consumption in the protection of intestinal tract. *Hybrid Webinar on Safe & Healthy Nutrition: insights into sports and ageing* – Faculty of Pharmacy of the University of Porto.

Pinho, S. C. M., Faria, M., Sobral, M. M. C., Casal, S., Alves, R., Cabrita, A. R. J., Fonseca, A. J. M., & Ferreira, I. M. P. L. V. O. (6-7th May 2021). Semi-dynamic gastric digestion of conventional versus pasture-based milk: fatty acids release and oxidative stability. *Virtual International Conference on Food Digestion* – Online.

Poster communications

Pinho, S. C. M., Faria, M. A., Melo, A., Pinto, E., Almeida, A. Alves, R., Cabrita, A. R. J., Fonseca, A. J. M., & Ferreira, I. M. P. L. V. O. (18th September 2020). Protective role of skim milk on intestinal oxidative stress and inflammation. *VII Annual Workshop for the Doctoral Program in Animal Science* – ICBAS, School of Medicine and Biomedical Science of the University of Porto.

Pinho, S. C. M., Meireles, P., Matos, E., Cabrita, A. R. J., Ferreira, I. M. P. L. V. O., & Fonseca, A. J. M. (18th September 2018) Modulating the protein profile and technological value of milk, emphasizing on casein enrichment through a nutritional approach. *V Annual Workshop for the Doctoral Program in Animal Science* – ICBAS, School of Medicine and Biomedical Science of the University of Porto.

ACKNOWLEDGEMENTS

Firstly, I would like to sincerely recognize my supervisor Professor António Fonseca for believing in me and always encouraged me to move forward. Thank you for advising me into applying to the Ph.D..

My deepest gratitude is to my co-supervisor Professor Isabel Ferreira. Her scientific guidance, motivation, and patience throughout these years made possible this thesis.

A special thanks to my industrial supervisor Eng. Rui Alves for the great support. I was very lucky to have had his help in the last three years of this journey.

My gratitude is immense to Doctor Miguel Faria, my unofficial supervisor. His approachability and interest were crucial for the development of this thesis.

I am so thankful to Professor Rita Cabrita for the fruitful discussions, encouragement, and motivation.

A sincere thanks to Professor Susana Casal for the concern demonstrated during the development of part of the experimental work, as well as for sharing scientific knowledge and enthusiasm.

Many thanks to Armindo Melo for the patience, guidance, and wisdom.

My deep appreciation to Edgar Pinto, Madalena Sobral, and Zita Martins for the scientific support and assistance in the development of parts of the experimental work.

Heartfelt thanks to Doctor Margarida Maia for the wise and comforting words.

I also gratefully acknowledge the sympathy and encouraging words of Sílvia Azevedo, Eulália Mendes, and Doctor Sara Cunha.

Great thanks to Olga Viegas, Mafalda Ribeiro, Marta Silva, Helena Ramos, Joana Costa, Caterina Villa, Liliana Grazina, Soraia Sá, Carolina Monteiro, Rebeca Cruz, Carla Teixeira, Anabela Costa, Liliana Espírito Santo, Joana Correira, Anabela Borges, Maria Antónia Nunes, Susana Machado, and Júlio Machado for creating an inspiring working environment.

Very warm thanks to my Ph.D. colleagues Ana Rita Pedro, Cátia Mota, Joana Fernandes, Liliana Cordeiro, Raquel Rodrigues, and Denis Meehan for the companionship.

A big thank-you to Cristina Xavier, Teresa Pinho, and Catarina Silva for being a source of kindness and strength.

A particular recognition to Dr. Pedro Meireles, Dra. Ana Canhola, Eng. Hilário, Pedro Maia, and Doctor Elisabete Matos for helping me in the Santa Cruz do Bispo Male Prison.

My special thanks go to all the people who, not being mentioned here, crossed with me in ICBAS-UP, FFUP, and Santa Cruz do Bispo Male Prison, and made my days' worth.

Last in the sequence, but not least in importance, thank you so much to my father and sister for the unconditional love.

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RESUMO

O leite é um alimento importante para uma alimentação humana saudável, pois é uma fonte de proteínas de elevada qualidade, vitaminas e minerais essenciais, e uma grande diversidade de ácidos gordos, devendo fazer parte da alimentação diária. No entanto, muitos guias alimentares aconselham a ingestão de leite com baixo teor de gordura preferencialmente ao leite gordo, pois este é rico em ácidos gordos saturados associados a efeitos negativos na saúde. Encontra-se disponível comercialmente numa grande variedade de designações em relação à sua origem geográfica e sistema de produção, sendo fatores conhecidos por modificar o perfil de nutrientes do leite. A associação entre o leite e a saúde é geralmente focada no seu perfil nutricional, no entanto, nos últimos anos, a investigação científica tem avançado do estudo dos nutrientes individuais para a bioatividade do leite como uma matriz completa. Especificamente, o epitélio gastrointestinal tem um papel central na relação dos alimentos com a saúde, desempenhado as funções de digestão e absorção, e participando ativamente como barreira física e imunitária.

Neste contexto, o principal objetivo da presente tese foi estudar o impacto da diferente origem geográfica e sistemas de produção na composição do leite e consequentes efeitos no processo de digestão e na saúde gastrointestinal. Em primeiro lugar, foi avaliada a lipólise gástrica e a oxidação lipídica do leite UHT gordo de diferentes sistemas de produção (leite convencional: a alimentação é normalmente à base de forragens conservadas e alimentos concentrados; e leite de pastagem), seguindo um protocolo semi-dinâmico de digestão gástrica *in vitro*. Posteriormente, foi estudado o impacto de digeridos *in vitro* de leite UHT 0% gordura, leite UHT gordo convencional e leite UHT gordo de pastagem na resposta celular no epitélio gástrico (monocamada de células NCI-N87), através da avaliação da expressão de ácido ribonucléico mensageiro (ARNm) de enzimas antioxidantes e moléculas inflamatórias. Por fim, foi avaliado o efeito do leite UHT magro de duas regiões geográficas portuguesas distintas (continente e Açores) na prevenção do aumento das espécies reativas de oxigénio e da apoptose no epitélio intestinal (co-cultura de células Caco-2/HT-29) e formação de óxido nítrico em macrófagos (RAW264.7) após digestão gastrointestinal *in vitro* estática.

As amostras de leite de dois sistemas de produção distintos apresentavam diferenças no perfil em ácidos gordos, nomeadamente o leite gordo de pastagem continha menor percentagem de ácidos gordos saturados e maior percentagem de ácidos gordos poliinsaturados do que o leite gordo convencional. Após a digestão gástrica *in vitro* semi-dinâmica, C8:0, C18:2n-6, C18:3n-3, e CLA-*cis*9*trans*11 livres apresentaram-se mais elevados no leite de pastagem comparado com o leite convencional ($P < 0,05$). Relativamente à oxidação lipídica, observou-se que o leite de pastagem apresentou uma

maior percentagem de formação de dienos conjugados comparado com o leite convencional ($P < 0,05$). Quando avaliada a resposta do epitélio gástrico à disponibilidade de ácidos gordos livres, um importante mecanismo homeostático das células, os resultados mostraram que a exposição repetida do modelo NCI-N87 de epitélio gástrico aos digeridos de leite aumentou a expressão de ARNm de catalase ($P < 0,05$), o que traduz uma resposta antioxidante específica para equilibrar o catabolismo dos ácidos gordos para a produção de energia. Em relação ao efeito do leite na expressão de moléculas inflamatórias, o leite gordo induziu maior expressão de ARNm de NF-κB p65 e IL-1β do que o leite 0% gordura ($P < 0,05$); não foram observadas diferenças entre o leite gordo convencional e o leite gordo de pastagem ($P > 0,05$). Após o tratamento com os digeridos de leite, foi adicionalmente aplicado um estímulo inflamatório com interferão-γ no modelo NCI-N87 de epitélio gástrico. As amostras de leite não influenciaram a expressão de NF-κB p65 e IL-1β nas células NCI-N87 estimuladas com interferão-γ ($P > 0,05$), indicando que o consumo regular de leite não aumenta subsequentes processos inflamatórios.

Relativamente às amostras de leite de duas regiões geográficas portuguesas distintas (duas de Portugal continental e duas dos Açores), estas apresentavam diferenças na sua quantidade relativa em lisina e fenilalanina e na concentração em diversos microminerais ($P < 0,05$). Os resultados mostraram também que a exposição do modelo Caco2/HT-29 de epitélio intestinal aos digeridos de leite magro levou à neutralização do aumento do nível de espécies reativas de oxigénio na co-cultura Caco2/HT-29 após exposição a uma baixa dose de peróxido de hidrogénio ($P < 0,05$), bem como à atenuação da formação de óxido nítrico nas células RAW264.7 estimuladas com lipopolissacarídeo ($P < 0,05$). No entanto, os digeridos de leite magro não protegeram a viabilidade da co-cultura Caco2/HT-29 após exposição a uma alta dose de peróxido de hidrogénio ($P > 0,05$). Não houve diferenças na bioatividade do leite magro, como matriz completa, entre as amostras ($P > 0,05$), apesar das diferenças na composição nutricional.

O presente trabalho mostrou que os sistemas de produção e as características geográficas afetam os aspetos nutricionais da digestão do leite pelo ser humano, uma vez que foram observadas diferenças na hidrólise relativa dos ácidos gordos entre os amostras de leite UHT gordo convencional e de pastagem. Em relação à bioatividade do leite, o leite UHT gordo induziu inflamação epitelial gástrica e o leite UHT magro preveniu o desequilíbrio redox nas células intestinais. A capacidade do leite UHT gordo para induzir inflamação epitelial gástrica foi semelhante entre as amostras de leite convencional e de pastagem, assim como o potencial do leite UHT magro para prevenir o desequilíbrio redox nas células intestinais foi semelhante entre as amostras de leite de Portugal Continental e dos Açores. Em conclusão, esta tese contribui para uma melhor compreensão da relação entre a composição do leite e consequentes efeitos no equilíbrio do epitélio gastrointestinal, o que

ajudará a orientar o setor dos lacticínios na procura de novas formas de promoção do leite na saúde pública.

Palavras-chave: leite de consumo, função gastrointestinal, bioatividade da matriz láctea, simulação da digestão humana

ABSTRACT

Milk is an important dietary source of high-quality proteins, essential vitamins and minerals, and an abundant variety of fatty acids for humans, being its intake often recommended on a daily basis to a healthy diet. Nevertheless, many dietary guidelines include messages to select low-fat milk over whole milk due to its richness in saturated fatty acids, which are linked to negative health effects. In retail outlets, a wide variety of milk labels regarding feeding production system and geographic origin is available, and these factors are known to affect milk nutrients profile. The association between milk and health is generally focused on its nutrients profile, however, in the last few years, nutrition research has shifted from the study of individual milk nutrients effects to the bioactivity of milk as a complete matrix. Specifically, the gastrointestinal epithelium plays a central role in the relationship between food and health, as it is involved in digestion and absorption, and is a physical and immunological active barrier.

In this context, the main objective of the present thesis was to advance the understanding of the impact of different geographic origins and feeding production systems on milk composition and consequent effects on human digestive process and the gastrointestinal health. Firstly, it was studied the susceptibility of UHT whole conventional (i.e., no management label; the majority is from housed cows offered conserved forages and concentrate feeds) and pasture-based labelled milks to gastric lipolysis and lipid oxidation using a semi-dynamic *in vitro* digestion protocol. Then, it was evaluated the impact of *in vitro* digested UHT fat-free, whole conventional, and whole pasture-based milks on cellular response of gastric epithelium (NCI-N87 monolayer) through the assessment of the messenger ribonucleic acid (mRNA) expression of antioxidant enzymes and inflammatory molecules. Lastly, it was assessed the effect of UHT skimmed milk from two distinct Portuguese geographic regions (mainland and the Azores), subjected to static *in vitro* gastrointestinal digestion, to neutralize increased reactive oxygen species and protect against apoptosis on intestinal epithelium (Caco2/HT-29 model), as well as to attenuate nitric oxide production by macrophages (RAW264.7 model).

Milk samples from different feeding production systems presented differences on fatty acids profile, namely whole pasture-based milk had lower relative content in saturated fatty acids and higher relative content in polyunsaturated ones than whole conventional milk. After the semi-dynamic *in vitro* digestion, free C8:0, C18:2n-6, C18:3n-3, and CLA-*cis*9*trans*11 were higher in pasture-based compared with conventional milk ($P < 0.05$). Regarding the lipid oxidation, it was observed that pasture-based milk had higher percentage of formation of conjugated dienes than the conventional ($P < 0.05$). The response of gastric epithelium to fatty acids availability is an important homeostatic mechanism and results showed that the

repeated exposure of NCI-N87 monolayer to *in vitro* digested milks up-regulated mRNA expression of catalase ($P < 0.05$), which constitutes a specific antioxidant response to accommodate the catabolism of fatty acids for energy production. In relation to the impact of milk on expression of inflammatory molecules, whole milk induced higher mRNA expression of NF-κB p65 and IL-1β than fat-free milk ($P < 0.05$); no differences were observed between the whole conventional and the pasture-based milk ($P > 0.05$). After milk digesta treatment, the NCI-N87 monolayer was stimulated with interferon-γ, which is a pro-inflammatory cytokine. Milk samples had no effect in the expression of NF-κB p65 and IL-1β of interferon-γ-stimulated NCI-N87 monolayer ($P > 0.05$), indicating that regular whole milk intake will not enhance subsequent inflammatory processes.

Regarding to the milk samples from two distinct Portuguese geographic regions (two from mainland Portugal and two from the Azores), they showed differences in the relative amount of lysine and phenylalanine and in the concentration of several microminerals ($P < 0.05$). Results showed that *in vitro* digested skimmed milk led to the neutralisation of increased reactive oxygen species level in the Caco2/HT-29 co-culture upon exposure to low-dose hydrogen peroxide ($P < 0.05$), as well as the attenuation of nitric oxide formation in the model lipopolysaccharide-stimulated RAW264.7 macrophage cells ($P < 0.05$). However, *in vitro* digested skimmed milk showed no protection against apoptosis in the Caco2/HT-29 co-culture exposed to a high-dose of hydrogen peroxide ($P > 0.05$). No differences in the bioactivity of skimmed milk, as a complete matrix, were observed among the four samples ($P > 0.05$), despite differences in nutritional composition.

The present work showed that geographic features and feeding production systems affect the nutritional aspects of human digestion, since it was observed differences in the relative release of individual fatty acids between UHT whole conventional and pasture-based milk samples. Regarding bioactivity, UHT whole milk induced gastric epithelial inflammation and UHT skimmed milk prevented intestinal cellular redox imbalance. The capacity of UHT whole milk to induce gastric epithelial inflammation was similar between the UHT whole conventional and the pasture-based samples, as well as the potential of UHT skimmed milk to prevent intestinal cellular redox imbalance was similar between the samples from mainland Portugal and the Azores.

In conclusion, this thesis contributes to a better understanding of the relationship between milk composition and the gastrointestinal health, which will help the dairy sector searching for new ways to promote milk in public health.

Keywords: retail milk, gastrointestinal function, milk matrix bioactivity, simulated human digestion

GENERAL INTRODUCTION

The present thesis was performed in an industrial setting, under the partnership of Soja de Portugal – a group that operates in the agro-food industry. In the European Union, dairy is the second biggest agricultural sector, after the vegetable and horticultural plant sector and before cereals, representing more than 12% of total agricultural output (Augère-Granier, 2018). Milk production is projected to grow at 0.5% per year from 2021 to 2031 in the European Union (to 162 million tons by 2031), being nutritional aspects and functionality the drivers of dairy demand (EC, 2021). In Portugal, the sector also represents 12% of agricultural production and dairy products represent 15% of total food industry output, being the most important subsector of food industry in terms of output value (Católica Porto Business School, 2020). Milk is the main dairy product consumed in Portugal; specifically, in 2020 the consumption of milk was 72.7 kg out of 118.8 kg of dairy products per inhabitant (INE, 2021).

Milk production can be described regarding two distinct cow feeding systems: indoor-feeding based on silage, hay, and concentrate feed that is predominant in Europe and North America, or pasture-based systems, which remain dominant in Ireland and New Zealand (Schwendel et al., 2017; Magan, Kelly, & McCarthy, 2021). Diet is an important factor affecting milk composition, specifically fatty acids profile, but other on-farm level factors such as breed, genetic variation within breed, stage of lactation, health, and season also affect its composition (Alothman et al., 2019; Benbrook et al., 2018). Retail samples represent a mixture of milk from a wide variety of farms, but regional agricultural practices and geographic features affect milk composition independently of the specific farm factors (Schwendel et al., 2015).

Milk consumption is recommended on a daily basis by over half of the dietary guidelines with messages about dairy (27 of 51 countries, according to the study of Herforth et al., 2019), because it is a source of several essential vitamins and minerals, and high-quality proteins. Nonetheless, dairy guidance is often accompanied by messages to prefer dairy products low in fat to whole dairy products as 70% of bovine milk fatty acids are saturated, which is linked to negative health effects (Comerford et al., 2021). The effect of milk fat on human health continues to require research, as it is apparent that its biological effects (similarly to what occurs in other complex foods) cannot be predicted by their content in a specific group of nutrients without considering its detailed composition, as well as the other groups of nutrients (Astrup et al., 2020).

Fatty acids are important sources of energy and can be involved in cell signalling processes, which affect gene expression and health (Chen, Michalak, & Agellon, 2018). The major saturated fatty acid in milk is C16:0 (MacGibbon & Taylor, 2006); this molecule has been

shown to promote the up-regulation of expression of cyclooxygenase-2 and several key pro-inflammatory cytokines (Calder, 2015). Notwithstanding, milk, despite being an important source of C16:0, comprises a heterogeneous group of saturated fatty acids, including short- and medium-chain fatty acids, branched-chain fatty acids, and odd-chain fatty acids (Astrup et al., 2020). Furthermore, fatty acids are not randomly distributed among milk fat glycerides and triacylglycerol structure influences the metabolic fate of its fatty acids (Michalski et al., 2013). In addition, the composition of milk fat is driven partially by cow's diet. Indeed, pasture feeding has been demonstrated to increase the content of beneficial fatty acids (e.g., C18:3n-3 and conjugated linoleic acids), while reducing the content of unhealthy ones (e.g., C16:0 and C18:3n-6) (Alothman et al., 2019; Benbrook et al., 2018).

Amino acids are essential to synthetize proteins in the organism and all essential ones are present in milk proteins in relatively high amounts (Gorska-Warsewicz, Rejman, Laskowski, & Czeczotko, 2019). Moreover, milk proteins are recognized sources of bioactive peptides (Pereira, 2014). In the human gastrointestinal tract, digestive enzymes generate peptides that may exert antimicrobial, antioxidative, anti-inflammatory, antihypertensive, or immunomodulatory effects on the major body systems, namely the digestive, cardiovascular, and immune systems (Boutrou, Henry, & Sanchez-Rivera, 2015; Tagliazucchi, Helal, Verzelloni, & Conte, 2016).

Although nutrients are essential for human health, the association between foods and health, focused on individual nutrients, has been considered a reductionist approach, because not all nutrients and non-nutritional compounds within foods have been fully studied, as well as there are synergistic and/or antagonistic interactions between components within complex foods (Tapsell, Neale, Satija, & Hu, 2016; Thorning et al., 2017). Currently, it has been proposed that research on the pointed relation should consider both the effects of whole food alongside with the individual nutrients (Mozaffarian, Rosenberg, & Uauy, 2018). Specifically, understanding the way different food nutrients are released in the gastrointestinal tract and the outcome of food digesta in the human digestive system is increasingly a topic of interest for nutrition and health researchers (Sensoy, 2021).

The demonstration of a relationship between food and health is thus a crucial issue in nutrition research. For that, direct nutritional intervention should be used as a gold standard, but these studies, as well as studies with animals, might generate ethical issues, depending on the study design (OECD, 2018). *In vitro* gastrointestinal models can overcome some of these difficulties and reduce the resources required. Attention has increasingly been focused on the development of *in vitro* digestion models and the setup of combined *in vitro* digestion and cellular models (Giromini, Cheli, Rebucci, & Baldi, 2019; Lu, Li, & Huang, 2019).

The main objective of the present thesis was to advance the understanding of the impact of different geographic origins and feeding production systems on milk composition and consequent effects on human digestive process and gastrointestinal health using *in vitro* models that simulate human digestion combined with gastrointestinal epithelial cellular assays.

To achieve the main objective, three specific objectives were established:

1. Follow gastric lipolysis and lipid oxidation of homogenized UHT whole conventional (i.e., no feeding management label) and pasture-based labelled milks by using the semi-dynamic digestion INFOGEST protocol (Figure i.1);
2. Study the impact on gastric epithelium of repeated exposure to homogenized UHT whole conventional, whole pasture-based and fat-free milks, after semi-dynamic *in vitro* digestion and simulating two different gastric luminal conditions – normal and inflammatory (Figure i.2);
3. Evaluate the potential of UHT skimmed milk from mainland Portugal and the Azores (after static *in vitro* gastrointestinal digestion) to neutralize the increase of reactive oxygen species and attenuate nitric oxide production, as well as its potential cytoprotective effect in the intestinal epithelium, and the role of minerals, free amino acids, and peptides (Figure i.3).

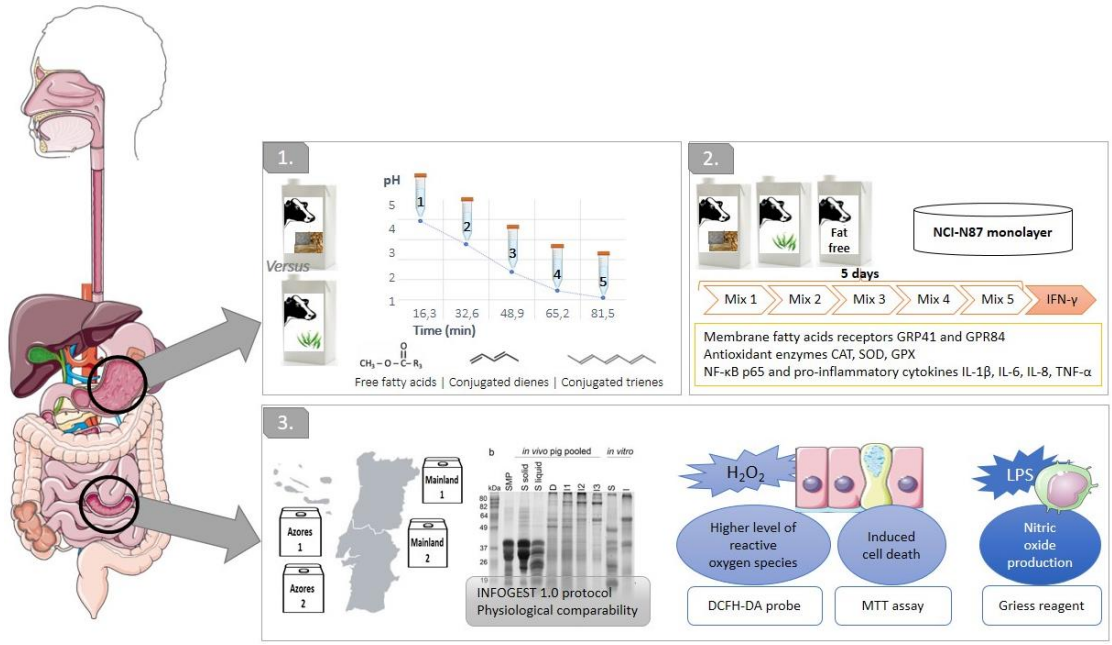

Figure i. Schematic summary of the three specific objectives of this thesis.

In the context of the SANFFED programme, the present thesis pretended to strength the ability of working with all food and agriculture actors for promotion of economic, social, and environmental development on behalf of the 2030 Agenda for Sustainable Development (Goal 17 – Partnership for the goals).

THESIS OUTLINE

This thesis was divided into three parts and four chapters that come out after a **General introduction** and **Thesis outline** and before **Concluding remarks and future perspectives** and **References** (references cited in **Chapter 2, 3, and 4**, which correspond to the articles published in this thesis, were not included in this section). Figure ii shows a schematic overview of the organization of this thesis.

Part I contains **Chapter 1 – State of the art** that presents a comprehensive overview about milk composition, functional role of its fat and protein fractions, and guidance for its consumption. Moreover, it is presented the role played by stomach and intestine in milk fat and protein digestion, the effects of milk nutrients within gastrointestinal tract, and summarizes the gastrointestinal models that replicate aspects of the human digestive system. To finish, it exposes the current lack of research on the functional effects of food as a complex matrix, not as a sum of nutrients, in order to a better understanding of the nutrition-health interface. The use of author-derived abbreviations was avoided in the body of this chapter.

Part II includes **Chapter 2, 3, and 4**, which correspond to the articles published in this thesis. Each chapter contains its own introduction, materials and methods, results, discussion, conclusion paragraph, and list of references. Author-derived abbreviations were defined in brackets after their first mention in the body of each article.

- **Chapter 2 – Explore gastric lipolysis and lipid oxidation of conventional versus pasture-based milk by a semi-dynamic *in vitro* digestion model** is focused on the application of the semi-dynamic INFOGEST protocol to study the differences in gastric behaviour of homogenized UHT whole conventional and pasture-based milks.
- **Chapter 3 – Gastric epithelial response to milk fat using the semi-dynamic INFOGEST digestion model coupled with NCI-N87 cells** studies the impact of homogenized UHT whole conventional, whole pasture-based and fat-free milks (after semi-dynamic *in vitro* digestion) on modulation of messenger ribonucleic acid (mRNA) expression of genes related to membrane fatty acids receptors, antioxidant enzymes, and inflammatory cytokines on gastric epithelium (using long-term testing) and simulating two different gastric luminal conditions – normal and inflammatory.
- **Chapter 4 – Effect of skimmed milk on intestinal tract: Prevention of increased reactive oxygen species and nitric oxide formation** is focused on the potential of skimmed milk from mainland Portugal and the Azores (after static *in vitro* gastrointestinal digestion) to neutralize high level of reactive oxygen species and attenuate nitric oxide production, as well as its potential cytoprotective effect in

intestinal epithelium, and the protective role of minerals, free amino acids, and peptides.

Part III contains **Chapters 5 – Overall discussion** that presents associations between the way different milk nutrients are released in the gastrointestinal tract and the outcome of milk digesta in the gastrointestinal epithelium and on health promotion/disease prevention. The use of author-derived abbreviations was avoided in the body of this chapter.

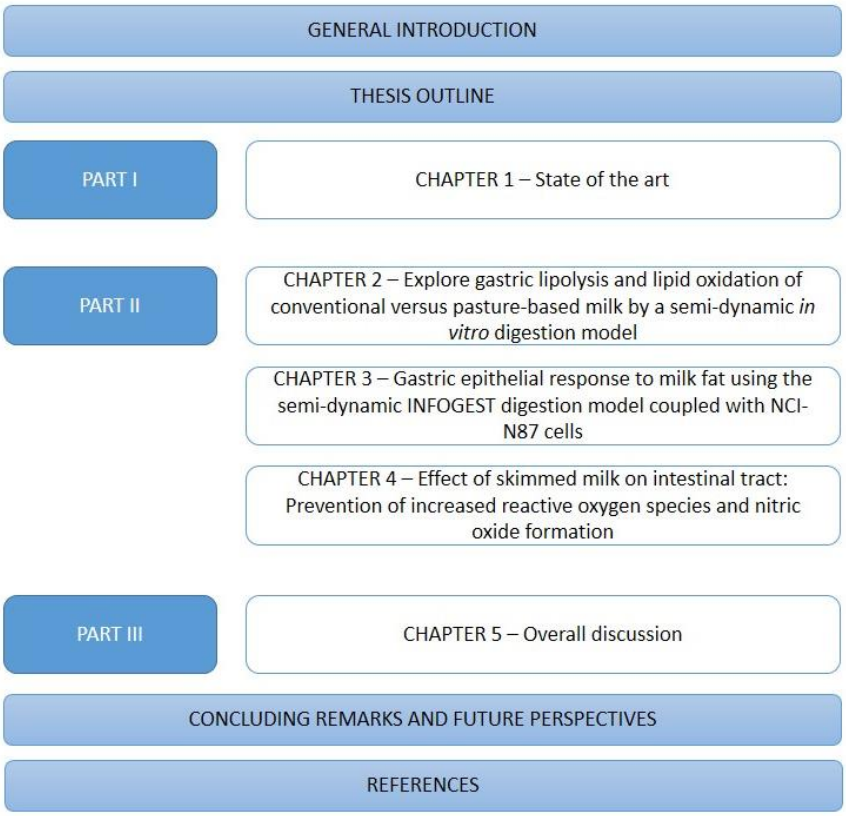

Figure ii. Thesis organization schematic overview.

PART I

CHAPTER 1 – State of the art

1.1. Milk: origin, compositional standards, fat and protein detail

As an agricultural product, “milk” is “the produce of the milking of one or more cows”; “as regards milk, the animal species from which the milk originates shall be stated, if it is not bovine” (Regulation EU No 1308/2013; EP, 2013). Therefore, from now one “cow’s milk” will be referred as “milk”. In Europe, at present, about 40% of milk is consumed in liquid form (i.e., “drinking milk”) (OECD & FAO, 2020). “Drinking milk” has established fat and protein content. It contains “a minimum of 2.9% (m/m) of protein for milk containing 3.5% (m/m) of fat or an equivalent concentration in the case of milk having a different fat content”. According to the Regulation (EU) No 1308/2013 (EP, 2013), “drinking milk” types include:

- raw milk: milk that has not been subjected to heat treatment above 40 °C or treatment having equivalent effect;
- whole milk: heat-treated milk with a fat content of at least 3.5% (m/m) (i.e. standardised whole milk); or heat-treated milk whose fat content has not been altered since the milking stage (i.e., non-standardised whole; minimum fat content of 3.5% (m/m));
- semi-skimmed milk: heat-treated milk whose fat content has been reduced; contains between 1.5% (m/m) and 1.8% (m/m);
- skimmed-milk: heat-treated milk from which fat has been removed to less than 0.5% (m/m)”.
- "… % fat": heat-treated milk not complying with fat content requirements of whole milk, semi-skimmed milk or skimmed milk. Fat content is on the packaging in form of "…% fat".

Milk production is predominantly founded on indoor farming and cow’s diet is based on silage, hay and concentrate feed throughout all seasons (Schwendel et al., 2017). Pasture-based milk production systems are possible in geographic areas of abundant rainfall that allows for consistent grass growth throughout the majority of the year (Magan, Kelly, & McCarthy, 2021). In Portugal, permanent pastures are a defining element in the Azores Islands, which climate is subtropical oceanic with mild temperature and rainfall all year long (Morais, Teixeira, Rodrigues, & Domingos, 2018).

Retail samples represent a mixture of milk from a wide variety of farms and thus other on-farm level factors than cow’s diet such as breed, genetic variation within breed, stage of lactation, health, and season also affect milk composition. However, food standards, regional agricultural practices, and geographic features (e.g., climate and altitude) affect milk composition independently of the specific farm factors (Schwendel et al., 2015). Therefore, the regulations of European Union and government laws such as Regulation

(EU) No. 2016/304 “Heumilch/Haymilk/Latte fieno/Lait de foin/Leche de heno” (EP, 2016a), Regulation (EU) No. 2018/848 “Organic” (EP, 2018), and Regional Legislative Decree No. 22/2016/A “Açores/Azores” (DR, 2016) can alter milk fatty acid and amino acid compositions.

At present, there are no legal requirements for detailed nutritional composition of milk from different production systems, but a study commissioned by the European Parliament supported that the feeding regime of dairy cows is reflected in the milk fatty acid composition. For instance, a cow’s diet containing fresh forage will result in a comparatively high content of omega-3 fatty acids in milk. According to the authors, a higher content of fresh forage is used in the feed of cows on organic production and total omega-3 fatty acid levels were found in 56% higher concentrations in organic than conventional milk. Specifically, C18:3n-3 was found in 69% higher concentration, C20:5n-3 was found in 67% higher concentration, and C22:5n-3 was found in 45% higher concentration. Furthermore, C18:1n-7 and C18:2-*cis*9,*trans*11 were found in 66% and 24% higher concentrations in organic than conventional milk (EP, 2016b).

1.1.1. Milk fat: fatty acids and its functional role

Milk fat is regarded as the most complex of all natural fats, because contains more than 400 different fatty acids with a variety of structures (Pereira, 2014). The diversity arises from the fatty acids synthesized *de novo* in the mammary gland (short- and medium-chain fatty acids), fatty acids derived from rumen bacteria (odd- and branched-chain fatty acids), fatty acids from feed lipids (C16 and C18 fatty acids) and fatty acids from the ruminal biohydrogenation of feed unsaturated fatty acids (MacGibbon & Taylor, 2006). The majority of these acids are present in trace quantities (<0.01% w/w) and only about 15 fatty acids are present at 1% w/w or higher (Mansson, 2008). A list of the major fatty acids in milk is presented in table 1.1.

Table 1.1. Major fatty acids in milk fat. Values were retrieved from the work of MacGibbon & Taylor, (2006).

		Composition % (w/w)	
	Common Name	**Typical**	**Range[a]**
C4:0	Butyric	3.9	3.1-4.4
C6:0	Caproic	2.5	1.8-2.7
C8:0	Caprylic	1.5	1.0-1.7
C10:0	Capric	3.2	2.2-3.8
C12:0	Lauric	3.6	2.6-4.2
C14:0	Myristic	11.1	9.1-11.9
C14:1	Myristoleic	0.8	0.5-1.1
C15:0	-	1.2	0.9-1.4
C16:0	Palmitic	27.9	23.6-31.4
C16:1	Palmitoleic	1.5	1.4-2.0
C18:0	Stearic	12.2	10.4-14.6
C18:1-*cis9*	Oleic	17.2	14.9-22.0
C18:1-*trans*	-	3.9	
C18:2n-6	Linoleic	1.4	1.2-1.7
C18:2-*trans*	Conjugated Linoleic acids	1.1	0.8-1.5
C18:3n-3	α-Linolenic	1.0	0.9-1.2
-	Minor acids	6.0	4.8-7.5

[a]Range of values for dairying season.

Fatty acids are the primary source of energy for cells. However, a high-fat diet is linked to negative effects on health (Astrup et al., 2020). Specifically, it has been demonstrated that a high-fat diet increases oxidative stress and dysfunctional mitochondria in several organs (Tan et al., 2018). Moreover, substantial evidence has suggested that saturated fatty acids induce pro-inflammatory signalling (Calder, 2015). The most important saturated fatty acid from a quantitative point of view is C16:0, accounting for about of 30% of the total fatty acids (Table 1.1). Results from an intervention study in humans showed that increased plasma C16:0 was associated with higher concentrations of circulating pro-inflammatory cytokine IL-6 (Dominguez-Lopez et al., 2022). However, different structures of saturated fatty acids appear to have differing effects on inflammatory processes. The shortest saturated fatty acid in milk fat is C:4 and no other common lipid source contains this fatty acid (Michalski et al., 2013). This fatty acid is a source of energy to the gut mucosa, but also shows a protective role in processes of inflammation in the intestine, acting through short-chain fatty acid-binding receptors (Portincasa et al., 2022). Other milk saturated fatty acids, such as odd (e.g., C15:0 and C17:0) and branched chain fatty acids (e.g., iso C15:0, iso C17:0,

anteiso-C15:0, and anteiso-C17:0) can also be potentially beneficial in reducing inflammation (Abdoul-Aziz, Zhang, & Wang, 2021).

Approximately 30% of the fatty acids in milk are unsaturated (Mansson, 2008). The most important unsaturated fatty acid from a quantitative point of view is C18:1, accounting for about of 20% of the total fatty acids (Table 1.1). Diets rich in this fatty acid have beneficial effects in inflammatory-related diseases and Howe, Burke, O'Reilly, McGillicuddy, & Costello (2022) hypothesized that a monounsaturated fatty acid-rich diet might mitigate inflammation despite obesogenic conditions. Milk also contains C18 *trans*-fatty acids in result of incomplete biohydrogenation of the unsaturated dietary lipids in the rumen. Dominant *trans* fatty acids from natural food sources are C18:1-*trans*11 and conjugated linoleic acid C18:2-*cis*9,*trans*11 is the main isomer (MacGibbon & Taylor, 2006). Conjugated linoleic acids may reduce the production of pro-inflammatory mediators in the human body (Chisaguano et al., 2014). The main poly-unsaturated fatty acids are C18:2n-6 and C18:3n-3 accounting for 1.4 and 1.0% of the total fatty acids (table 1.1). The fatty acids C18:2n-6 and C18:3n-3 must be provided in the human diet, because mammals lack the desaturases that are needed to introduce double bonds at the Δ12 and Δ15 positions of fatty acids. The presence of these fatty acids in milk results from hydrogenation processes in the rumen by specific microorganisms that modify feed-derived C18 fatty acid (Buitenhuis et al., 2019).

Some studies showed that non-conventional (e.g. pasture-based/extensive and organic) milks have higher nutritional value and thus healthier than conventional ones, but it is questionable whether non-conventional milks contain some valuable nutrients in sufficient amounts that support human health (EP, 2016b). For instance, a cow's diet containing fresh forage will result in a comparatively high content of C18:3n-3 in milk (about 50% higher in retail HTST organic than conventional milk; Capuano, Gravink, Boerrigter-Eenling, & van Ruth, 2015). However, milk is considered not to contribute significantly to the overall daily intake of C18:3n-3 and thus the advantage of consuming non-conventional milk would be negligible (van Valenberg, Hettinga, Dijkstra, Bovenhuis, & Feskens, 2013). Similarly, even though the concentration of conjugated linoleic acids is higher in organic milk (25-30%), extrapolation from animal studies indicates that conjugated linoleic acids content in milk should be at least 4 times as high as the average milk content to exert beneficial effects in humans (Capuano et al., 2015).

According to the study commissioned by the European Parliament "Human health implications of organic food and organic agriculture", at this point, it is not possible to conclude the existence of health benefits from a change from milk obtained by conventional systems to a milk with higher content of some valuable nutrients (EP, 2016b).

1.1.2. Milk protein: amino acids and its functional role

Milk is considered the most near perfect protein source, because contains all the nine indispensable amino acid in adequate amounts (chemical score = 95%), a non-truncated protein-digestibility corrected amino acid score of 120% (Pellegrino, Masotti, Cattaneo, Hogenboom, & de Noni, 2013), and a true ileal digestibility of 94-99% (Tomé, 2021).

The amino acid composition of milk is primarily influenced by genetic factors, as the amino acids transferred to the mammary gland to synthetize caseins, β-lactoglobulin, and α-lactalbumin (around 90% of total milk nitrogen) may be genetically determined. However, the relative abundance of low-molecular-weight nitrogen compounds in milk serum phase may be influenced by dairy feeding system, modulating overall milk amino acids composition (Magan et al., 2019; O'Callaghan et al., 2018).

Amino acids have an enormous biological relevance, because they are key precursors for *de novo* synthesis of proteins, which serve as structural support, enzymes, hormones, neurotransmitters, and initiators of cellular death (Wu, 2009). Beyond their participation as building blocks of proteins, amino acids directly participate in metabolic pathways, enzymatic processes, and redox reactions (Estevez et al., 2020).

Redox control by amino acids can have an important role in biological systems, because of their abundance within cells (between 0.1–40 mM, without counting the protein-containing amino acids) and high capacity to quench free radicals through hydrogen donation, or neutralize and break the oxidative chain reactions through electron transference (Aluko, 2015; Stadtman & Levine, 2003). Recently, Guidea, Zagrean-Tuza, Mot, & Sarbu (2020) compared the antioxidant activity of the twenty proteinogenic amino acids using many different analytical methods based on chemical reactions, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical-scavenging activity, metal chelating activity, superoxide radical-scavenging activity, nitric oxide scavenging activity, ferric reducing antioxidant power, cupric ion reducing antioxidant activity, and chromium reducing antioxidant capacity. The authors showed that mostly of the amino acids containing heteroatoms (cysteine, glutamic acid, aspartic acid) or aromatic moieties (tyrosine, phenylalanine, and tryptophan) in their side chain had a significant DPPH radical-scavenging ability, while for ABTS radical-scavenging activity they behave in opposition to this, except cysteine, which had much stronger antioxidant reactivity towards ABTS compared to DPPH. At the same concentration, asparagine, histidine, phenylalanine, tryptophan, and tyrosine have shown the highest superoxide radical-scavenging activity (over 80%) and a potential for inhibition of nitric oxide over 51%. Cysteine (96.72%) and tryptophan (85.30%) showed the highest reducing ferric reducing antioxidant power among all the twenty proteinogenic amino acids.

Regarding cupric ion reducing antioxidant activity and chromium reducing antioxidant capacity of amino acids, cysteine showed the highest values (95.77%) in case of cupric ion reducing antioxidant activity, and arginine, glutamine, leucine, and tryptophan in the case of chromium reducing antioxidant capacity of amino acids. Overall, cysteine and tryptophan present the highest antioxidant activity, followed by tyrosine, asparagine and arginine; the amino acids with least antioxidant activity are alanine, methionine, threonine, and valine (Guidea et al., 2020).

Moreover, specific domains of milk proteins are particularly resistant to gastrointestinal proteases, as well as endo-/exo-peptidases from intestinal brush border membranes, suggesting that some peptides are able to cross the gut mucosal barrier and may reach target organs of the body, where could exert antioxidant effect (Power, Jakeman, & FitzGerald, 2013). Current knowledge on the circulating levels of these peptides in the blood is limited. However, recently, Caira et al. (2022) identified milk-derived peptides with antioxidant properties (deduced from the Milk Bioactive Peptide Database) circulating in the plasma of three healthy volunteers who drunk 250 mL of pasteurized milk from the local market after a 10-days washout using a two-step purification procedure prior to nanoliquid chromatography-electrospray-tandem mass spectrometry analysis (Table 1.2). According to this study, most of milk-derived peptides with antioxidant properties circulating in the plasma belong to β-casein. Inter-individual variations were observed and they could be due to genetic, gut microbiota, and health status differences (Nongonierma & FitzGerald, 2015).

Table 1.2. Milk-derived peptides with antioxidant properties detected in blood samples. Data retrieved from the work of Caira et al. (2022).

Peptide	Volunteer	Protein
APSFSDIPNPIGSENSE	1	α_{S1}-casein
AYFYPEL	1	α_{S1}-casein
FALPQYLK	1	α_{S2}-casein
YPFPGPIPN	1, 2, 3	β-casein
VYPFPGPIPN	1	β-casein
IHPFAQTQ	1	β-casein
YQEPVLGPVR	1	β-casein
AVPYPQR	1, 2	β-casein
VLPVPQK	1	β-casein
SQSKVLPVPQKAVPYPQ	1, 2	β-casein
FPKYPVEPF	1	β-casein
HKEMPFPKYPVEPFTESQ	1	β-casein
ARHPHPHLSFM	1	κ-casein
INNQFLPYPYYAKPA	1	κ-casein

The presupposed health benefits of antioxidant properties of milk-derived peptides has been investigated in the context of physical exercise, which is associated with oxidative processes. Particular attention is paid to whey proteins even though most of the bioactive peptides present in the digestive tract of adult humans and animals are released from caseins (Boutrou et al., 2013). Whey protein is a key ingredient of hydrolysates designed for nutritional applications in sports nutrition. Lollo et al. (2014) showed that the supplementation of elite soccer players with hydrolysed whey protein (0.5 g protein/kg of body mass/ day) for 12 weeks resulted in the decrease of *in vivo* markers of oxidative stress and tissue damage creatine kinase (-42%) and lactate dehydrogenase (-30%).

Milk proteins are also an interesting source of minerals. Indeed, hydroxyl groups of some amino acids in casein can be esterified with phosphoric acid, which binds calcium, magnesium and some complex salts of sodium, potassium, chloride, sulfate, and citrate. Other minerals, for example, zinc, iron, molybdenum, copper, and selenium, are present in enzymes, many of which are concentrated in the milk fat globule membrane (Goulding, Fox, & O'Mahony, 2020). Dairy feeding system has little influence on the mineral composition of milk, especially phosphorus and calcium as these minerals are complexed with caseins (van Hulzen et al., 2009). However, diet can influence iodine, manganese, molybdenum, selenium, zinc, and cobalt concentrations in milk (Rey-Crespo, Miranda, & Lopez-Alonso, 2013).

1.2. Dietary guidance for the consumption of dairy products

To promote healthy eating habits and lifestyles, many countries or regions developed food-based dietary guidelines (also known as dietary guidelines) (Fischer & Garnett, 2016). Dairy is a foundational food group in many food-based dietary guidelines. Indeed, dairy is its own food group in 64% of food guides worldwide, and 75% of countries include dairy in their key messages (Herforth et al., 2019).

Currently, most dairy messaging in food-based dietary guidelines recognize milk as an important dietary source of calcium, vitamin D, iodine, potassium, and/or high-quality protein. Nonetheless, dairy guidance is often accompanied by messages to prefer low fat and fat-free products (Table 1.3; Comerford et al., 2021; Herforth et al., 2019). These recommendations are based on nutrient-focused guidelines that associate milk consumption with chronic diseases, because of its high content in unhealthy saturated fatty acids. Increasing evidence, however, suggests that dairy fat can be neutral or beneficial for human health (Lordan, Tsoupras, Mitra, & Zabetakis, 2018). Indeed, some health authorities have changed their advices on consumption of full-fat dairy even though most

food-based dietary guidelines continue to recommend limiting milk fat intake. For instance, in 2019, Australian Heart Foundation announced that healthy Australians could consume full-fat dairy products without concerning about increasing their risk of heart disease or stroke (The New Daily, 2019). Thus, in the last few years, nutrition research has shifted between an individual nutrient approach and a food-based approach.

Table 1.3. Examples of messages conveyed by dietary guidelines encouraging low-fat forms of milk. Dairy messages were retrieved from the work of Herforth et al. (2019) and the work of Comerford et al. (2021).

Country	Message
Chile	"To strengthen your bones, consume low-fat and low-sugar dairy three times per day."
Grenada	"Change from whole milk to reduced fat, from reduced fat to low fat and from low fat to fat-free or non-fat or skimmed milk."
Iceland	"It is recommended to choose the most low-fat, unsweetened or low-sugar dairy products without sweeteners."
Italy	"Drink a cup of milk or yogurt every day, preferably choosing the partially skimmed one, which maintains its content in calcium and proteins."
Lebanon	"As full-fat milk and dairy products can substantially contribute to the intake of total fat and saturated fat, low-fat and fat-free versions should be selected."
Netherlands	"Low-fat and semi-skimmed dairy are preferable to full-fat dairy."
Slovenia	"Use milk and dairy products that are low in fat."
Sweden	"Choose low-fat, unsweetened products enriched with vitamin D."

In Portugal, the food guide is represented as a food wheel (Portuguese: A Roda dos Alimentos; English: Food wheel guide) divided into segments representing food groups. Milk and dairy products should constitute 18% of daily caloric intake, which corresponds to two or three servings per day. A serve from the dairy food group is a cup (250 mL) of milk, a cup (200 mL) of drinkable yoghurt or a cup and a half (200 g) of yoghurt, two slices (40 g) of cheese, one-quarter of a cup (50 g) of fresh cheese, and half of a cup (100 g) of whey cheese. There are no messages regarding fat content of dairy products (FCNAUP & Instituto do Consumidor, 2003).

In recent years, growing concern on the detrimental environmental impact of the way food is produced and consumed led to the concept of "sustainable healthy diets". The guiding principles of FAO and WHO (2019) for sustainable healthy diets recommends the intake of moderate amounts of dairy. The EAT-*Lancet* Commission on healthy diets from sustainable food systems recommends 250 (0-500) g/day of whole milk or derivative equivalents (Willett

et al., 2019). Regarding the way food is produced, FAO and WHO (2019) referred that territorial diets, such as the Mediterranean Diet (moderate consumption of dairy foods), have a pivotal role in supporting the transition towards more sustainable agriculture and food systems (FAO & WHO, 2019). The EAT-*Lancet* Commission on healthy diets from sustainable food systems did not compare specific food production systems (e.g., organic *versus* conventional), because diets can disguise diversity of contexts and available solutions (Willett et al., 2019).

1.2.1. Chronic diseases, oxidative stress, and inflammation

Some food-based dietary guidelines refer metabolic factors or chronic diseases as explicit reasons for limiting dairy fat intake (Herforth et al., 2019). High intakes of this macronutrient plays a great role in generating hypertension, overweight/obesity, hyperglycemia, and hyperlipidemia, which are the four key metabolic changes that underlie chronic diseases (GBD, 2016), the leading cause of human disability and death worldwide (FAO & WHO, 2019).

The cellular pathology that contributes to the pathogenesis of chronic diseases involves oxidative stress and inflammation, which are closely related pathophysiological processes, one of which can be easily induced by the other (Biswas, 2016; Pena-Oyarzun et al., 2018). Reactive oxygen species (free radicals such as superoxide radicals and hydroxyl radicals, and non-radical oxidants such as hydrogen peroxide and hypochlorous acid) are normal byproducts of cellular metabolism, but their generation can be stimulated by metabolism of saturated free fatty acids (Han, 2016).

Cells present mechanisms of protection against excess of reactive oxygen species, namely endogenous enzymatic (e.g. superoxide dismutases, glutathione peroxidase, glutathione-reductase, catalase, and superoxide reductases) and endogenous non-enzymatic antioxidants (e.g. glutathione, thioredoxin, and melatonin), which act on the metabolism of reactive species (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014). In addition, exogenous antioxidants obtained from the diet (e.g. vitamin C, vitamin E, zinc, copper, manganese, iron, selenium, and carotenoids) have an important role in removing free radicals from the system (Marcadenti, 2015). However, a high reactive oxygen formation combined with antioxidant-deficiency can lead to excess of reactive oxygen species produced (i.e., oxidative stress), which can either oxidize biomolecules or trigger many inflammatory signalling cascades, such as factor nuclear NF-kB and cyclooxygenase-2 (Biswas, 2016).

Additionally, fatty acids can act directly on the inflammatory process (sterile inflammation). Current evidence suggests that saturated fatty acids and lipopolysaccharides share the

same inflammatory signalling pathway as toll-like receptor 4 and can recruit neutrophils and macrophages, whose functions include the production of reactive oxygen species (Rocha, Caldas, Oliveira, Bressan, & Hermsdorff, 2016).

Taken together, it is evident that excessive saturated fat consumption can promote oxidative stress and inflammation. Nevertheless, other nutrients of the diet appear to combat oxidative stress and inflammation such as vitamin C, vitamin E, zinc, copper, manganese, iron, selenium, and carotenoids. Therefore, food choices can exacerbate or decrease the level of oxidative stress and inflammation (Tan, Norhaizan, & Liew, 2018).

1.3. Milk digestion and physiological effects

Understanding the way different food nutrients are released in the gastrointestinal tract and the outcome of food digesta in the human digestive system is increasingly a topic of interest for nutrition and health researchers (Sensoy, 2021). Gastrointestinal epithelium is the first tissue that interacts with nutrients and other compounds released from food. In detail, gastrointestinal cells are important for absorption of food constituents, secretion of enzymes involved in lipid and carbohydrate absorption, secretion of gut hormones, local immune responses, and provide a barrier against the entry of pathogens, toxins, and allergens from the gastrointestinal lumen to the systemic circulation (Greenwood-Van Meerveld, Johnson, & Grundy, 2017).

1.3.1. Digestion of milk fat

Milk fat is particularly susceptible to gastric lipolytic activity. Indeed, milk fat is rich in short- and medium-chain fatty acids, which are predominantly located at the *sn*-3 position in triacylglycerol molecule, and gastric lipase exhibits preference for cleavage at the *sn*-3 position and specificity to short- and medium-chain fatty acids (Bauer, Jakob, & Mosenthin, 2005; Michalski et al., 2013). Moreover, the proportion of C8:0 and C10:0 at *sn*-3 position in milk triacylglycerol is negatively correlated with the amount of C16:0, the most abundant fatty acid in milk (22-35% of total fatty acids) (Tzompa-Sosa, van Aken, van Hooijdonk, & van Valenberg, 2014). The relative amount of C16:0 in milk fat depends greatly on cow nutrition, as up to 60% of total milk fatty acids (w/w) originate from feed (≥C18 fatty acids and also some C16:0) (MacGibbon & Taylor, 2006). Specifically, milk fat from cows on pasture has lower proportion of C16:0, whereas milk fat from conventional dairy management (i.e., housed cows offered conserved forages and concentrate feeds, which usually incorporate calcium soap from palm oil) has a higher proportion of C16:0 (Alothman et al., 2019; O'Callaghan et al., 2016). Therefore, differences may occur on lipolysis extent

of conventional an pasture-based milks, but existent studies do not use gastric lipase, nor mimic gradual acidification, nor emptying (Van Hekken, Tunick, Ren, & Tomasula, 2017).
Moreover, gastric bioaccessibility of unsaturated fatty acids can be lower than that expected as stomach is considered a bioreactor of lipid peroxidation (Gorelik, Ligumsky, Kohen, & Kanner, 2008). Oxidative degradation of fatty acids is related to the fatty acids composition and to the concomitant presence of other components exerting a pro-oxidant or antioxidant behaviour (Gorelik et al., 2008; Nieva-Echevarria, Goicoechea, & Guillen, 2020). Antioxidants present in milk are mostly from the fresh forages or from the antioxidant supplementation of concentrate feeds provided to cows (Castillo, Pereira, Abuelo, & Hernandez, 2013). Particularly, milk from pasture-based systems may present a higher concentration of specific antioxidants, namely plant secondary metabolites, which would have a preventive effect on the oxidative deterioration of polyunsaturated fatty acids (Krizova, Krestakova, Dadakova, & Kasparovsky, 2021).
Overall, between 5−40% of dietary fat is hydrolysed in the stomach (Michalski, 2009). Fatty acids are important sources of energy and can be involved in cell signalling processes, which affect gene expression and health (Chen et al., 2018). Nonetheless, information concerning the impact of milk on gastric cells function is scarce. Indeed, in metabolically active organs (e.g. liver, heart, and brain), fatty acids exhibit signalling functions, regulating pathways essentially intertwining metabolism and inflammation through free fatty acids receptor-mediated signalling (Kimura, Ichimura, Ohue-Kitano, & Igarashi, 2020; Miyamoto et al., 2016). The stomach is a less active organ, but it was demonstrated that gastric cells also express membrane fatty acids receptors (Widmayer et al., 2015). Consequently, a long-term intake of dietary fat is supposed to be associated with up-regulation of membrane receptors for free fatty acids in the stomach (Nunez-Salces, Li, Christie, & Page, 2020), thereby potentially mediating the effects of free fatty acids on epithelial metabolism and inflammation.
The partially digested fat moves into the small intestine and free fatty acids reduce further food intake by eliciting satiety signals. For instance, fatty acids up to 12 carbons are absorbed directly into the portal circulation and transported to the liver for rapid β-oxidation, thus being readily used as a source of energy and resulting in faster satiety (St-Onge & Jones, 2002). The fatty acids with a chain length greater than 12 carbons are effective releasers of the cholecystokinin, a hormone recognized to suppress appetite, and therefore of paramount importance for healthy weight management (Rehfeld, 2021).
Pancreatic lipase, colipase, other esterases, and bile salts complete milk lipolysis in the intestine (Bauer et al., 2005; Michalski et al., 2013). Pancreatic lipase-colipase is responsible for the major lipolysis in the intestine, having activity towards the fatty acids in the primary (*sn*-1,3) positions of the di- and triacylglycerols. The fatty acids in the *sn*-2

positions are subjected to a non-enzymatic isomerization to 1-monoacylglycerols under the alkaline conditions of the small intestine, which makes them available for enzymatic hydrolysis, allowing full triacylglycerol hydrolysis (Brownlee et al., 2010).

1.3.2. Digestion of milk protein

Digestion of proteins starts in stomach, where pepsin cleaves at the phenylalanine or leucine bond, which results in peptides of varying size with only a very small amount of free amino acids (Egger et al., 2019; Tapal & Tiku, 2019). Indeed, by using skimmed milk powder and applying the INFOGEST 1.0 protocol, Egger et al. (2019) reported about 0.38% of free amino acids during the gastric phase.

In small intestine, pancreatic enzymes continue the digestion of peptides: trypsin cleaves after an arginine or lysine amino acid, and chymotrypsin acts at phenylalanine, tyrosine, or tryptophan bonds. At the same time, brush border enzymes such as aminopeptidase and dipeptidase, hydrolyse peptides to amino acids as well as di- and tripeptides (Egger et al., 2019; Tapal & Tiku, 2019). Egger et al. (2019) observed an increase of free amino acids from 0.38% to 50.2% during the intestinal phase. Moreover, digestion of milk proteins lead to the release of numerous peptides in the intestinal endpoint; Egger et al. (2019) reported that the peptide pattern of *in vitro* digestion showed a good approximation to the *in vivo* study with pigs.

Intestinal epithelium is constantly exposed to triggers that stimulate reactive oxygen species generation, and thus great focus has been placed on antioxidant peptides. Indeed, Tagliazucchi et al. (2016) studied the production and nature of antioxidant compounds released during the *in vitro* digestion of protein from whole, semi-skimmed and skimmed milk. The *in vitro* digested whole milk sample showed the lowest antioxidant capacity (38% of ABTS radical-scavenging activity in the low molecular weight fraction), while the percentage of ABTS radical-scavenging activity in the low molecular weight fraction increased to 79 and 90% in semi-skimmed and skimmed milk samples, respectively, according to the increase in the proteolysis degree. Gastrointestinal proteases release some amino acid sequences and free amino acids with antioxidant activities that were inactive in the intact proteins. Regarding the antioxidant capacities of the peptides constitutive amino acids, tryptophan was the amino acid with the highest ABTS value followed by cysteine and tyrosine.

The antioxidant protective effects of peptides within the intestinal lumen have been investigated against H_2O_2-induced oxidative stress using human intestinal epithelial cells. Among milk antioxidant peptides, caseinophosphopeptides have received much attention as they can sequester prooxidant metals and scavenge free radicals (Power et al., 2013).

Specifically, García-Nebot, Cilla, Alegría, & Barberá (2011) observed that caseinophosphopeptides obtained from casein subjected to simulated gastrointestinal digestion protected the Caco-2 cells against H_2O_2-induced oxidative damage by preserving cell viability, increasing intracellular content of reduced glutathione, and inducing catalase enzyme activity.

Free amino acids are also involved in physiological radical reactions within the intestinal lumen. Indeed, Katayama & Mine (2007) pre-treated Caco-2 cells with amino acids before the stimulation with H_2O_2 and observed that H_2O_2-induced IL-8 secretion from Caco-2 cells (an indicator of tissue oxidative stress) was inhibited by cysteine, valine, isoleucine, leucine, tryptophan, histidine, lysine, and alanine.

Although individual milk peptides obtained by simulated gastrointestinal of milk proteins, as well as amino acids, present antioxidant protective effects in Caco-2 cells, further research on the potential health effects of milk alongside the more traditional approach of studying the health effects of its single nutrients is required (Giromini et al., 2019). Moreover, regional characteristics such as local breeds or local genetic variation within breed may influence the amino acid composition of milk digesta (Schwendel et al., 2015) and thus its antioxidant and other health protective effects.

1.4. Gastrointestinal models

Human gastrointestinal models replicate aspects of the human digestive system to have information as accurate as possible about food disintegration, releasing of nutrients, and interactions between the food and gastrointestinal barrier (Mackie, Mulet-Cabero, & Torcello-Gomez, 2020). *In vitro* digestion models have been widely used, because involve fewer ethical restrictions and enables high-throughput studies than animal models (Mulet-Cabero et al., 2020). Moreover, the setup of combined *in vitro* digestion and cellular models has been increasingly implemented to assess the interaction of digestion metabolites with the gastrointestinal cells (Giromini et al., 2019; Lu et al., 2019).

Milk consumption is recommended on a daily basis, being the gastrointestinal tract repeatedly exposed to milk nutrients. Nonetheless, studies assessing the impact of exposure of gastrointestinal cells to digestion metabolites is generally based on short durations (i.e., less than 5 days) (Pfaller et al., 2001; Giromini et al., 2019). Therefore, the use of appropriate cellular models of gastrointestinal epithelium and long-term testing enables a better understanding of the impact of digested milk without fractionation in gastrointestinal epithelium.

1.4.1. In vitro *digestion models*

Several *in vitro* digestion models have been proposed to physiologically mimic the gastrointestinal digestive process. These are very diverse, from single static systems to multi-compartmental dynamic complex equipment (Lucas-Gonzalez, Viuda-Martos, Perez-Alvarez, & Fernandez-Lopez, 2018). Static *in vitro* digestion models are much simpler and less expensive than the complex dynamic computerized versions, not accessible to all laboratories, and thus widely used in many fields of food and nutritional sciences (Luiking, Abrahamse, Ludwig, Boirie, & Verlaan, 2016). Various static *in vitro* digestion protocols are available in literature, making difficult the comparison of experimental data between laboratories. In 2014, an international harmonized three-phase static *in vitro* digestion protocol (oral, gastric and intestinal) was published (i.e., INFOGEST 1.0 protocol), aiming to improve the comparability of experimental data (Minekus et al., 2014). The biological relevance of this digestion protocol has been validated using pigs as animal model and it is suited to study protein hydrolysis at the gastric and intestinal endpoints (Egger et al., 2017). Nevertheless, INFOGEST 1.0 protocol is not appropriated to study lipid digestion. Gastric lipase was not included in the gastric phase of this protocol due to unavailability of relevant commercial gastric lipases (Mackie et al., 2020). In 2019, INFOGEST 1.0 protocol was updated and the revised protocol (INFOGEST 2.0 protocol) included gastric lipase, commercially available as rabbit gastric extracts (Brodkorb et al., 2019). However, this model suffers from some limitations. During gastric digestion, a gradual change of pH occurs on stomach, which influences lipolysis, because gastric lipase is pH-dependent and presents optimum hydrolysis activity between pH 4 and 6 (Mackie et al., 2020).

Recently, the gastric phase of INFOGEST 2.0 protocol was updated and now mimics the transient nature of gastric secretions (semi-dynamic INFOGEST protocol) (Mulet-Cabero et al., 2020). In contrast to static versions, specific type of equipment is needed to perform the semi-dynamic INFOGEST protocol, such as a digestion vessel, devices that control enzymes and fluid secretion, and a pH meter (Mackie et al., 2020). Nonetheless, the intestinal phase remains similar to the INFOGEST 1.0 and INFOGEST 2.0 protocols and is performed individually with each aliquot emptied from the gastric phase after neutralization to pH 7. Static versions recommend a constant pH of 7 during the intestinal phase, while the pH varies from about 7 to between 6 and 5 in the duodenum due to acidic gastric emptying (Mulet-Cabero et al., 2020). Thus, the semi-dynamic INFOGEST protocol only provides physiologically relevant data regarding the gastric phase of lipid digestion (Alegría, Garcia-Llatas, & Cilla, 2015). A comparison of the current available standardized protocols is presented in Table 1.4.

Table 1.4. Comparison of the current available standardized protocols.

	INFOGEST 1.0	**INFOGEST 2.0**	**Semi-dynamic INFOGEST**
Oral phase	Static phase 1:1 food:simulated salivary fluid (w:w)	Static phase 1:1 food:simulated salivary fluid (w:w)	Static phase 1:1 dry weight of food :simulated salivary fluid (w:w)
Gastric phase	Static phase 1:1 oral bolus:simulated gastric fluid (v:v) Enzymes: pepsin pH 3.0 (constant) Incubation time: 2h Gastric emptying: not included Equipment: not specified	Static phase 1:1 oral bolus:simulated gastric fluid (v:v) Enzymes: pepsin + gastric lipase pH 3.0 (constant) Incubation time: 2h Gastric emptying: not included Equipment: not specified	Dynamic phase 1:1 oral bolus:simulated gastric fluid (v:v) Enzymes: pepsin + gastric lipase Gradual acidification up to pH 2 (end of gastric phase) Incubation time based on the caloric content of the food Gastric emptying: 2 kcal/min (minimum of 3 gastric emptying aliquots) Equipment: digestion vessel, devices that control enzymes and fluid secretion, and a pH meter
Intestinal phase	Static phase 1:1 gastric chyme:simulated intestinal fluid (v:v) Incubation time: 2h	Static phase 1:1 gastric chyme:simulated intestinal fluid (v:v) Incubation time: 2h	Static phase 1:1 aliquots of gastric emptied chyme:simulated intestinal fluid (v:v) Incubation time: 2h each aliquot emptied from the gastric phase
Suitability	Protein digestion in gastrointestinal tract: the physiological relevance of the gastric and intestinal endpoints was demonstrated in a pig *in vivo* trial conducted by Egger et al. (2017)	Includes the use of gastric lipase, but it is not suitable for simulating digestion kinetics	Lipid digestion in stomach: this digestion method includes crucial kinetic aspects associated with the gastric phase of digestion

1.4.2. Cellular models

The lumen of the human gastrointestinal tract is bordered by epithelial cells that can be mimicked *in vitro* with models exhibiting unique characteristics of the human gastrointestinal functions. In order to mimic the human gastrointestinal epithelium, most of the assays so far developed employ immortalized cell lines, since the differentiation and proliferative capabilities of gastric and intestinal cells extracted from a living animal are difficult to maintain *in vitro* (Creff, Malaquin, & Besson, 2021; Yokobori et al., 2016).

Gastric epithelium is a complex structure folded into glands, which contain a wide variety of cells types, such as mucin-5ac-secreting surface mucous cells, mucin-6-secreting mucous neck cells, acid-secreting parietal cells, and enzyme-secreting chief cells (Dimaline & Varro, 2007). Although several gastric cancer cell lines are available (e.g. AGS, KATO-III, MKN-28 and NCI-N87 cells), only NCI-N87 cells show mixed functional markers of mucous neck cells and chief cells, as well as exhibits the capacity to form tight and coherent monolayers (Basque, Chenard, Chailler, & Menard, 2001).

Intestinal epithelium contains several cell types, including enterocytes, goblet cells, Paneth cells, and neuroendocrine cells, held together by tight junctions (Greenwood-Van Meerveld et al., 2017). The Caco-2 cell line has been widely used as a model of the intestinal epithelium due to its ability to differentiate into a monolayer with the properties of enterocytes (Lea, 2015). However, due to the presence of more than one prevalent cell type in the intestinal epithelium, some authors have proposed mixing enterocytes cells with goblet cells to mimic fully the intestinal epithelial lining. For instance, the intestinal 70/30 Caco2/HT-29 co-culture proposed by Ferraretto et al. (2018) constitute a versatile and suitable model to investigate intestinal epithelial functions.

1.5. Research gaps

From the previous comprehensive overview, the following gaps were identified:

- Geographic origin features and feeding production systems impart milk nutritional composition, however, at this point, it is not possible to conclude whether these differences influence gastrointestinal health;
- Research on *in vitro* gastric lipolysis and lipid oxidation of milk from different feeding systems is scarce and do not use physiomimetic methods;
- Information concerning the impact of whole milk on gastric epithelial inflammation is very limited and *in vitro* studies do not mimic long-term exposure;

- Literature also lacks information concerning the antioxidant effect of milk as a complete matrix in intestine using more physiomimetic models of intestinal epithelium.

The articles published in this thesis (**Part II – Chapter 2, 3, and 4)** were designed to address these research gaps.

PART II

CHAPTER 2 – Explore gastric lipolysis and lipid oxidation of conventional versus pasture-based milk by a semi-dynamic *in vitro* digestion model

CHAPTER 3 – Gastric epithelial response to milk fat using the semi-dynamic INFOGEST digestion model coupled with NCI-N87 cells

CHAPTER 4 – Effect of skimmed milk on intestinal tract: Prevention of increased reactive oxygen species and nitric oxide formation

CHAPTER 2 - Explore gastric lipolysis and lipid oxidation of conventional versus pasture-based milk by a semi-dynamic *in vitro* digestion model

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

pubs.acs.org/JAFC Article

Explore Gastric Lipolysis and Lipid Oxidation of Conventional versus Pasture-Based Milk by a Semi-dynamic *In Vitro* Digestion Model

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Explore gastric lipolysis and lipid oxidation of conventional versus pasture-based milk by a semi-dynamic *in vitro* digestion model

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Cite this: Pinho, S. C. M., M. Faria, S. Casal, M. M. C. Sobral, R. Alves, A. R. J. Cabrita, A. J. M. Fonseca, and I. M. P. L. V. O. Ferreira. Explore gastric lipolysis and lipid oxidation of conventional versus pasture-based milk by a semi-dynamic in vitro digestion model. *J. Agric. Food Chem.* **2021**, *69,* 14241-14249, https://doi.org/10.1021/acs.jafc.1c03150.

Abstract

Research on gastric lipolysis of commercial cow's milk with different fatty acid (FA) compositions is scarce. Gastric lipase exhibits specificity for the *sn*-3 chain position of triacylglycerols, whose structure is influenced by milk FA composition. Therefore, during gastric digestion of conventional (C) vs pasture-based (P) milk, differences may occur on lipolysis, which has impact on free FA available, influencing their absorption/metabolism rate and physiological hormonal responses. Those two milk types were subjected to the INFOGEST semi-dynamic digestion model. Five gastric emptying points were analyzed for oxidative degradation of polyunsaturated fatty acids (PUFA) and individual free FA. The relative release of medium-chain FA (C8:0−C12:0) was higher than that of longer-chain FA (C14:0−C18:0), and a linear increase in markers of PUFA oxidative degradation occurred along gastric digestion. Quantitatively, C8:0, C18:2n-6, C18:3n-3, and CLA*c*9*t*11 were higher ($P < 0.001$) in P milk when compared with C milk.

Keywords: UHT whole milk, cow's milk fat, human simulated gastric digestion, free fatty acid, conjugated dienes and trienes

2.1. Introduction

During gastric digestion, a gradual change of pH occurs on the stomach, which influences lipolysis because gastric lipase is pH-dependent and presents optimum hydrolysis activity between pH 4 and 6.[1] Therefore, simulation of this physiological condition is required to properly address fatty acid (FA) release and lipid oxidation during gastric digestion. The novel semi-dynamic model proposed by INFOGEST[2] mimics the transient nature of gastric secretions and emptying because it simulates the physiological changes in hydrochloric acid and enzyme secretions, being suggested as an upgrade of the widely used static method.[3,4] The semi-dynamic model is much simpler and less expensive than the complex dynamic computerized versions not accessible to all laboratories.[5]

Milk has been used as a model food for harmonization of *in vitro* digestion (IVD) models.[2,3] However, information about the gastric lipolysis of milks from the same species with different FA composition is limited and existent studies do not mimic gradual acidification nor emptying.[6] Moreover, the recommendation to use gastric lipase, commercially available as rabbit gastric extracts (RGE), was addressed only recently on the INFOGEST 2.0 protocol,[4] which hindered the previous study of milk lipolysis with the referred methods.

Human gastric lipase exhibits *sn*-3 stereopreference, allowing the release in the stomach of short-chain and medium-chain FA that are predominantly found at the *sn*-3 position of milk triacylglycerols (TAG).[7] Moreover, the TAG structure is influenced by milk FA composition.[8−10] For example, the proportion of C8:0 and C10:0 at the *sn*-3 position in milk TAG is negatively correlated with the amount of C16:0, the most abundant FA in milk (22−35% of total FA).[10] The proportion of C16:0 depends on the cow breed and nutrition,[11] as milk fat from cows on pasture (eating fresh forages) has a lower proportion, whereas milk fat from non-grazing cows (conventional dairy management, i.e., housed cows offered conserved forages and concentrate feeds) has a higher proportion of C16:0.[12,13] Moreover, fresh pasture feeding seems to beneficially alter the FA composition of milk with an increased concentration of polyunsaturated fatty acids (PUFA).[12]

During gastric digestion, oxidative degradation of FA can occur in addition to the hydrolytic process, being dependent on several factors related to FA composition and to the concomitant presence of other components exerting a prooxidant or antioxidant behavior.[14,15] In milk, the oxidative deterioration of FA is attenuated initially by antioxidants available in the milk serum and later in the oxidation process by lipophilic reducing constituents.[16] Antioxidants present in milk come mostly from the fresh forages or from the antioxidant supplementation of concentrate feeds provided to cows.[17] Particularly, milk from pasture-based systems may present a higher concentration of specific antioxidants, namely

plant secondary metabolites with a preventive effect on the oxidative deterioration of PUFA.[18]

This work aimed to apply the semi-dynamic *in vitro* model of INFOGEST described by Mulet-Cabero et al.[2] to study the gastric lipolysis and lipid oxidation of commercial cow's milks with different FA profiles since this model provides a good *in vivo−in vitro* correlation.[1] Two homogenized UHT whole milks (no management label and pasture-based labeled) were selected to assess the release of FA and formation of markers of oxidation in PUFA, taking into account the dynamics of pH and enzymes secreted at the stomach as well as the emptying. To the best of our knowledge, this work is pioneer in examining gastric behavior (lipolysis and oxidative degradation) of commercial cow's milk that present different FA profiles (PUFA-richer milk compared to a regular one). Milk fat gastric behavior is important since the pattern of free FA can affect its absorption and consequent metabolic responses in humans, such as satiety.[1]

2.2. Materials and methods

2.2.1. Selection of samples

Milk samples were obtained from retail outlets. Six homogenized UHT whole milk brands (three with no management label, brands 1−3, and three pasture-based-labeled, brands 4−6) were purchased in November 2020 to cover a wide variety of individual cows and farms. All samples were characterized concerning their FA profile and total antioxidant activity. Two brands (one of each label) were then selected based on their different balance of C16:0 and PUFA. Those samples were coded as C milk (no management label/conventional milk) and P milk (pasture-based milk).

2.2.2. Reagents and apparatus

Pepsin (P7012, 2144.3 units/mg, porcine origin) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit gastric extract (RGE15, lipase activity = 15.2 units/mg and pepsin activity = 431.4 units/mg) was purchased from Lipolytech (Marseille, France). Enzyme activities were determined according to Brodkorb et al.[4] Triundecanoin (C11:0 TAG) was acquired from Larodan AB (Solna, Sweden). Tricosanoic acid (C23:0), butylatedhydroxytoluene (BHT), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), fluorescein, and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were from Sigma-Aldrich (St Louis, MO, USA). Methyl tridecanoate (C13:0 methyl ester), acetic acid, and sulfuric acid (H_2SO_4) were from Fluka (St. Gallen, Switzerland). Fatty acid methyl ester

(FAME) standard mixtures and the aminopropylbonded phase cartridge (Discovery DSC-NH2, 500 mg, 3 mL) were obtained from Supelco Inc. (Bellefonte, PA, USA).

High-performance liquid chromatography (HPLC)-grade *n*-hexane was purchased from Merck (Darmstadt, Germany). Chloroform (Puriss p.a.), methanol (Puriss p.a.), 2-propanol (Puriss p.a.), diethyl ether (Puriss p.a.), and toluene (HPLC-grade) were from Riedel-de-Haën (Charlotte, NC, EUA). Isooctane (ACS) was acquired from Carl Erba (Milano, Italy). Ultrapure water purified by a Milli-Q gradient system (18.2 $m\Omega cm^{-1}$) from Millipore (Milford, DE, USA).

A 751 GPD Titrino dosing device (Metrohm AG, Herisau, Switzerland), a syringe pump (Model 100 Series) (KD Scientific Inc., MA, USA), a pH meter (BASIC 20+) (Crison, Barcelona, Spain), a vessel with a thermostat jacket (Metrohm AG, Herisau Switzerland), and a vessel lid with openings (Metrohm AG, Herisau Switzerland) were used in the gastric IVD assay. A UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) was used for the analysis of conjugated dienes (CD) and trienes (CT). In the oxygen radical absorbance capacity (ORAC) assay, a fluorescence microplate reader (Biotek, Winooski, VT, USA) was used.

For lipid extract preparation, a Nederman nitrogen sample concentrator (Helsingborg, Sweden) was used. FAME was analyzed by gas chromatography with flame ionization detection (GC-FID); a Chrompack CP 9001 chromatograph (Middelburg, Netherlands) equipped with a split/splitless injector system was used. FA separation was carried out on a J&W CP-Select FAME capillary column (50 m × 0.25 mm × 0.19 *μ*m; Agilent, Santa Clara, CA, USA).

2.2.3. In vitro *gastric semi-dynamic digestion*

Human simulated gastric digestion was performed according to the standardized semi-dynamic protocol described by Mulet-Cabero et al.[2] The macronutrient content described on nutritional labeling was used to calculate the conditions for simulation of gastric dynamics as indicated in the protocol: the C milk and the P milk had both 3.6% fat, 3.3% protein, and 4.9 or 5.0% carbohydrates. A real intake of 250 mL was considered and scaled down using the protocol's supplementary information. First, 15 mL of milk was mixed for 2 min at 37 °C with simulated salivary fluid (SSF): 1.44 mL of simulated salivary electrolyte fluid (eSSF) + 9.0 *μ*L of 0.3 mol/L $CaCl_2(H_2O)_2$ + 0.351 mL ultrapure water, without amylase in a ratio of dry weight of milk to SSF of 1:1 (w/v). Then, the oral phase was placed into a 70 mL glass v-form vessel thermo-stated at 37 °C containing 10% of the simulated gastric fluid (SGF): 1.176 mL of eSGF + 0.148 mL of 1.5 mol/L HCl + 0.8 *μ*L of 0.3 mol/L $CaCl_2(H_2O)_2$ + 0.168 mL enzyme solution (157.9 mg gastric lipase RGE15 + 4.7 mg pepsin). The amount of HCl

required was determined for both milks in test experiments prior to digestion to reach a pH of <2 at the final of the gastric phase. The remaining 90% of the SGF (13.608 mL of eSGF +1.48 mL of 1.5 mol/L HCl + 8.4 μL of 0.3 mol/L $CaCl_2(H_2O)_2$ + 1.512 mL of enzyme solution) were added at a constant ratio by separated devices. A polylactic acid printed paddle stirrer was used at 15 rpm for agitation following the protocol recommendations.

Five gastric emptying (GE) points of 6.72 mL each were performed every 16.30 min using a pipette with an end internal diameter of 2 mm. Before each sampling, the vessel content was homogenized with a syringe to collect a fraction representative of the whole gastric content since the structure of the coagulum from homogenized UHT-treated milks is fragmented.[19] The pH was measured in each emptied aliquot. A general scheme of the methodology is presented in Figure 2.1. Lipase activity was stopped by adding a chloroform/methanol mixture required for the lipid extraction[4,20] performed immediately after collection. The two samples were digested in duplicate on different days from different 1 L packages of a 6 × 1 L pack.

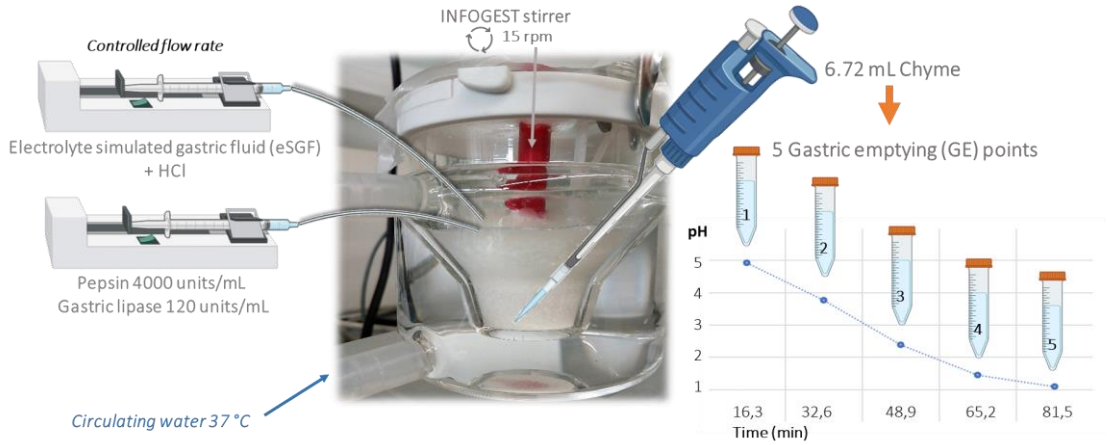

Figure 2.1. Schematic representation of the gastric phase of the INFOGEST semi-dynamic method, highlighting the titration vessel, the two pumps to add fluids and enzymes, and the printed stirrer set at 15 rpm. Five gastric emptying points were performed, and pH was measured in each one.

2.2.4. Analysis of products of lipolysis

2.2.4.1. Extraction of total lipids

Lipids were extracted from milk and digested samples using the Bligh and Dyer method,[20] according to Mulet-Cabero et al.[21] Milk samples were diluted with 1% NaCl solution before extraction and treated as the GE samples. For each 0.5 mL of sample, 0.5 mL of chloroform, 120 μL of internal standard in chloroform (60 μL of 13.5 mg/mL C11:0 TAG and 60 μL of 3 mg/mL C23:0 FA), and 1.25 mL of methanol were added. Then, 0.625 mL of chloroform and 0.625 mL of water with 1% NaCl were included, and samples were centrifuged at 3000

g for 10 min. The methanol−water layer was collected and treated with 3 mol/L HCl to reach a pH of <1.5. The extraction procedure was repeated,[22] and the organic phases were pooled and evaporated to dryness under a gentle stream of nitrogen.

2.2.4.2. Separation of free fatty acids (FFA)

The FFA were separated by solid-phase extraction using aminopropyl cartridges, according to Kaluzny et al.[23] The cartridges were first conditioned with 4 mL of *n*-hexane before the lipid extract in chloroform (<0.5 mL) loading onto the cartridge. Next, the column was eluted with 4 mL of chloroform:2- *n*-propanol (2:1 v/v) to separate all neutral lipids. The FFA fraction was then eluted with 4 mL of diethyl ether containing 2% acetic acid (v/v) to a new tube, and the solvent was further evaporated to dryness under a stream of nitrogen.

2.2.4.3. Preparation of FAME and GC-FID analysis

FA were converted into FAME using the acid transesterification process,[24] as described by Mulet-Cabero et al.:[21] 0.5 mL of toluene (containing 0.02% BHT) and 1 mL of methanol containing 2% H_2SO_4 (v/v) were added to lipid extracts. After vortex-mixing, tubes were placed in the oven at 50 °C during 15 h. After cooling down to room temperature, they were added with 1 mL of neutralizing solution (10.5 g $NaHCO_3$ and 34.55 g of K_2HCO_3 dissolved in 500 mL of ultrapure water), 1 mL hexane, and 10 *µ*L of 22 mg/mL C13:0 methyl ester in hexane. Samples were vortex mixed and centrifuged at 100 *g* for 5 min. The organic phase was transferred to a 2 mL vial, concentrated to 200 *µ*L, and further analyzed by GC-FID. For each sample, 1.0 *µ*L was injected in duplicate. Helium was used as carrier gas at 140 kPa. Temperatures of the injector and detector were 250 and 270 °C, respectively. The oven temperature was 120 °C, increasing to 220 °C at a rate of 3 °C/min. FAME standard mixtures were injected alongside with samples. The FA composition of milk (brands 1−6) was expressed as the percentage of total FAME present in these samples. Concerning the relative release of individual FA, FFA mass at the GE points (6.72 mL of chyme, FFA_{GEx}) was expressed as the percentage of the corresponding total FA mass in the ester form present in 15 mL of undigested milk ($EFA_{undigested\ milk}$):

$$\text{FA relative release (\%)} = FFA_{GEx}/EFA_{undigested\ milk} \times 100 \quad (1)$$

$EFA_{undigested\ milk}$ was calculated taking in consideration the total FA ($TFA_{undigested\ milk}$) and FFA quantified ($FFA_{undigested\ milk}$) in 15 mL:

$$EFA_{undigested\ milk} = TFA_{undigested\ milk} - FFA_{undigested\ milk} \quad (2)$$

All GE points were also analyzed for total FAME to determine their fat content. The mass of each major FA was estimated by the chromatographic area of C11:0 or C23:0. The FA C4:0 and C6:0 were not included due to their high volatily,[25] being partially lost during sample preparation.

2.2.5. Analysis of PUFA oxidation

To follow the development of lipid oxidation, extinction values were calculated from the absorbance of CD (233 nm) and CT (268 nm) measured in 10 mg of lipid extracts[26] of undigested milk or GE points dissolved in 4 mL of isooctane (i.e., standardized amount of fat). Results are expressed as the percentage of formation of CD and CT in each GE point relative to the undigested milk.

2.2.6. Analysis of the total antioxidant capacity of milk

The total antioxidant capacity was determined according to the AOAC official method 2012.23 (ORAC using fluorescein as the fluorescence probe).[27] Milk samples were prepared by applying the hydrophilic ORAC procedure for blood plasma or serum; the addition of diluted milk in phosphate buffer to fluorescein working solution and AAPH was previously used by Zulueta et al.[28] to determine hydrophilic ORAC plus lipophilic ORAC in milk. Trolox concentrations used on the calibration curve were in the 0−8.0 *μ*mol/L range, selected from a preliminary test to obtain a complete reaction in 35 min. All six milk samples were diluted to 3:1000 with phosphate buffer (pH 7.4) to reach a complete reaction at the same time of the standards. Results are expressed as *μ*M Trolox equivalents (TE).

2.2.7. Statistical analysis

The statistical analysis of the results was performed using SPSS software for Windows version 26 (Chicago, IL, USA). Results were expressed as mean ± standard deviation. The differences among normally distributed results of relative content of FA in brands 1−6 were assessed by one-way ANOVA followed by Tukey's *post hoc* test. An independent *t*-test for two samples was carried out for detecting differences between the C milk and the P milk means in each GE point regarding pH, FFA level, and CD and CT formation. For all comparisons, differences were considered significant at a *P*-value lower than 0.05. The graphs were built in GraphPad Prism version 7.05 for Windows (GraphPad Software, La Jolla, CA, USA).

2.3. Results and discussion

2.3.1. Selection of samples and characterization

A preliminary study of FA composition and total antioxidant capacity of six different commercial milks (three with no management label, brands 1−3, and three pasture-based labeled milks, brands 4−6) was conducted. The FA profile of milk samples is presented in Table 2.1. Milk brand 6 presented a distinct FA profile, namely a lower proportion of C16:0 and a higher proportion of PUFA ($P < 0.05$). The considerable differences in the FA profile observed among brands within the pasture-based group suggest flexibility within the label "pasture-based" regarding daily intakes of fresh forage as reported by Butler et al.[29] and Capuano et al.[30] Among milk brands with no management specifications, brand 2 had a similar content of macronutrients compared to brand 6 (information found in the nutritional labeling) and did not present considerable differences in the FA profiles compared to milks 1 and 3 (Table 2.1). Concerning the total antioxidant capacity, the lower ORAC value was observed for milk brand 4 (13,316 ± 1472 *μ*M TE) and the higher for milk brand 2 (15,013 ± 1107 *μ*M TE), while the ORAC value of milk brand 6 was 13,576 ± 1189 *μ*M TE. Nevertheless, the mean ORAC values were not statistically different ($P > 0.05$) for the different brands. This result is in line with the findings of Ellis et al.[31] and Smet et al.[16] who reported no significant differences in the content of selected vitamins of milks from cows under different feeding practices. Specifically, Ellis et al.[31] observed no significant differences in the *α*-tocopherol and *β*-carotene contents; Smet et al.[16] reported no significant differences for total ascorbic acid. Ellis et al.[31] suggested that the effect of feeding fresh forages may be less marked in the milk vitamin content than expected because, in general, higher levels of concentrate feeds (which are supplemented with synthetic vitamins) are provided to cows under conventional management, increasing its concentration in milk. Regarding other milk constituents with antioxidant properties that can differ depending on cow nutrition, studies have reported significant differences in the content of the isoflavone-derived metabolite equol.[32,33] Milk from cows grazing a pasture containing red clover present higher concentrations of equol as compared with milk from cows grazing on grass pasture or eating conserved forages. Nonetheless, in general, red clover is less abundant in natural pastures than in fields intended for the production of conserved forages.[18]

Thus, regarding FA composition and total antioxidant capacity, brands 2 and 6 were selected as representative of milk from cows under conventional (i.e., housed cows offered conserved forages and concentrate feeds) and pasture-based feeding systems and will be from now one referred to as C (conventional) milk and P (pasture) milk, respectively.

Table 2.1. Profile of selected fatty acids (expressed as % of total FAME) of different dairy milk brands (1−3: no management label, 4−6: pasture-based label)[a].

Fatty acid[1]	Brand 1[2]	Brand 2[2]	Brand 3[2]	Brand 4[2]	Brand 5[2]	Brand 6[2]
C8:0	1.42 ± 0.04	1.37 ± 0.11	1.39 ± 0.03	1.39 ± 0.07	1.40 ± 0.09	1.41 ± 0.12
C10:0	2.44 ± 0.11^{ab}	2.37 ± 0.03^{b}	2.47 ± 0.04^{ab}	2.54 ± 0.05^{a}	2.50 ± 0.02^{ab}	2.43 ± 0.11^{a}
C12:0	3.14 ± 0.10^{c}	3.06 ± 0.04^{c}	3.07 ± 0.05^{c}	3.42 ± 0.05^{b}	3.55 ± 0.03^{a}	2.93 ± 0.10^{c}
C14:0	10.8 ± 0.21	10.6 ± 0.06	10.5 ± 0.09	11.2 ± 0.13	11.0 ± 0.05	10.7 ± 0.31
C15:0	1.09 ± 0.06^{b}	1.08 ± 0.02^{b}	1.05 ± 0.05^{b}	1.10 ± 0.01^{b}	1.12 ± 0.02^{b}	1.26 ± 0.05^{a}
C16:0	33.6 ± 0.23^{a}	32.8 ± 0.15^{ab}	32.8 ± 0.10^{ab}	33.1 ± 0.07^{a}	32.1 ± 0.06^{b}	29.0 ± 0.27^{c}
C17:0	0.48 ± 0.01^{e}	0.52 ± 0.01^{cd}	0.50 ± 0.01^{bde}	0.53 ± 0.00^{bc}	0.54 ± 0.02^{bc}	0.64 ± 0.01^{a}
C18:0	10.2 ± 0.13^{b}	10.1 ± 0.04^{b}	10.0 ± 0.05^{b}	10.1 ± 0.05^{b}	10.1 ± 0.02^{b}	10.9 ± 0.20^{a}
C18:1*t*[3]	2.53 ± 0.13^{b}	2.67 ± 0.02^{b}	2.67 ± 0.06^{b}	2.69 ± 0.04^{b}	2.68 ± 0.04^{b}	3.91 ± 0.26^{a}
C18:1*c*9	21.7 ± 0.24^{ab}	22.4 ± 0.19^{a}	21.9 ± 0.11^{ab}	21.8 ± 0.15^{b}	21.6 ± 0.13^{b}	22.1 ± 0.22^{ab}
C18:2*t*[4]	0.80 ± 0.02^{bc}	0.70 ± 0.02^{c}	0.78 ± 0.06^{bc}	0.85 ± 0.04^{b}	0.89 ± 0.04^{b}	1.01 ± 0.08^{a}
C18:2n-6	2.38 ± 0.06^{ab}	2.25 ± 0.09^{b}	2.45 ± 0.03^{a}	2.43 ± 0.03^{a}	2.34 ± 0.02^{ab}	1.70 ± 0.12^{c}
C18:3n-6	0.027 ± 0.003	0.027 ± 0.003	0.026 ± 0.001	0.029 ± 0.002	0.027 ± 0.002	0.023 ± 0.005
C18:3n-3	0.37 ± 0.03^{b}	0.35 ± 0.06^{b}	0.36 ± 0.04^{b}	0.41 ± 0.02^{b}	0.42 ± 0.03^{b}	0.60 ± 0.03^{a}
C20:0	0.15 ± 0.00^{b}	0.16 ± 0.01^{ab}	0.17 ± 0.00^{ab}	0.19 ± 0.02^{a}	0.16 ± 0.02^{ab}	0.18 ± 0.02^{ab}
CLA-*c*9*t*11	0.58 ± 0.00^{bc}	0.51 ± 0.02^{d}	0.55 ± 0.01^{cd}	0.64 ± 0.06^{b}	0.61 ± 0.03^{bc}	1.50 ± 0.06^{a}
CLA-*t*10*c*12	0.052 ± 0.007^{b}	0.056 ± 0.007^{b}	0.062 ± 0.006^{ab}	0.084 ± 0.017^{a}	0.084 ± 0.008^{a}	0.071 ± 0.009^{ab}
C21:0	0.025 ± 0.017^{b}	0.024 ± 0.007^{b}	0.024 ± 0.001^{b}	0.030 ± 0.001^{ab}	0.028 ± 0.007^{ab}	0.040 ± 0.014^{a}
C20:2n-6	0.025 ± 0.006^{ab}	0.016 ± 0.003^{bc}	0.027 ± 0.006^{a}	0.018 ± 0.003^{bc}	0.016 ± 0.004^{c}	0.014 ± 0.002^{c}
C20:3n-6	0.016 ± 0.001^{ab}	0.015 ± 0.000^{b}	0.019 ± 0.003^{a}	0.015 ± 0.001^{bc}	0.016 ± 0.001^{b}	0.011 ± 0.001^{c}
C20:4n-6	0.062 ± 0.013^{a}	0.051 ± 0.010^{a}	0.047 ± 0.003^{ab}	0.033 ± 0.007^{bc}	0.034 ± 0.006^{bc}	0.025 ± 0.008^{c}

C22:0	0.057 ± 0.015	0.066 ± 0.002	0.063 ± 0.004	0.070 ± 0.001	0.073 ± 0.002	0.061 ± 0.008
C20:5n-3	0.041 ± 0.006^{bc}	0.034 ± 0.010^{c}	0.046 ± 0.007^{bc}	0.064 ± 0.006^{a}	0.056 ± 0.008^{ab}	0.050 ± 0.007^{abc}
C24:0	0.057 ± 0.009	0.052 ± 0.005	0.059 ± 0.003	0.060 ± 0.010	0.056 ± 0.005	0.065 ± 0.014
C22:5n-3	0.044 ± 0.008^{d}	0.052 ± 0.005^{cd}	0.047 ± 0.007^{d}	0.076 ± 0.007^{bc}	0.091± 0.011^{ab}	0.12 ± 0.020^{a}
SFA[5]	63.5 ± 0.89^{a}	62.3 ± 0.60^{a}	62.2 ± 0.19^{a}	63.6 ± 0.61^{a}	62.6 ± 0.23^{a}	59.6 ± 1.01^{b}
MUFA[6]	24.3 ± 0.16^{bc}	25.0 ± 0.19^{b}	24.6 ± 0.10^{bc}	23.7 ± 0.18^{c}	24.3 ± 0.10^{bc}	26.1 ± 0.21^{a}
PUFA[7]	4.39 ± 0.05^{c}	4.05 ± 0.15^{d}	4.41 ± 0.06^{c}	4.65 ± 0.06^{b}	4.58 ± 0.09^{bc}	5.13 ± 0.10^{a}

[α] Total FAME, in g/100 mL milk, was brand 1 = 3.38 ± 0.23, brand 2 = 3.53 ± 0.11, brand 3 = 3.45 ± 0.29, brand 4 = 3.44 ± 0.10, brand 5 = 3.61 ± 0.13, and brand 6 = 3.49 ± 0.12. [1]Values represent mean ± standard deviation of three replicates (each one injected in duplicate). [2]Probability of significant differences among treatment groups. NS = $P > 0.05$. [3]Sum of all *trans*-C18:1 FA. [4]Sum of all *trans*-C18:2 FA. [5]Saturated fatty acids, sum of C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, and C24:0. [6]Monounsaturated fatty acids, sum of C18:1*t* and C18:1*c*9. [7]Polyunsaturated fatty acids, sum of C18:2*t*, C18:2n-6, C18:3n-6, C18:3n-3, CLA-*c*9*t*11, CLA-*t*10*c*12, C20:2n-6, C20:3n-6, C20:4n-6, C20:5n-3, and C22:5n-3. [a-e]Significant differences within a row are indicated by different superscript letters.

2.3.2. Oxidative stability of milk fat during semi-dynamic gastric digestion

A linear increase was observed in the formation of CD and CT from the first GE point (pH ~4.90) to the last (pH < 2) (Figure 2.2) due to the low pH of gastric medium, which is an unavoidable pro-oxidant condition; therefore, higher oxidative degradation of PUFA occurred as gastric digestion progressed and pH decreased. Other unavoidable pro-oxidant condition in the stomach is the presence of oxygen and *in vitro* digestion experiments can be performed either in open vessels or in closed vessels with different air proportions; the latter is considered a better simulation of real conditions because less oxygen is available.[15]

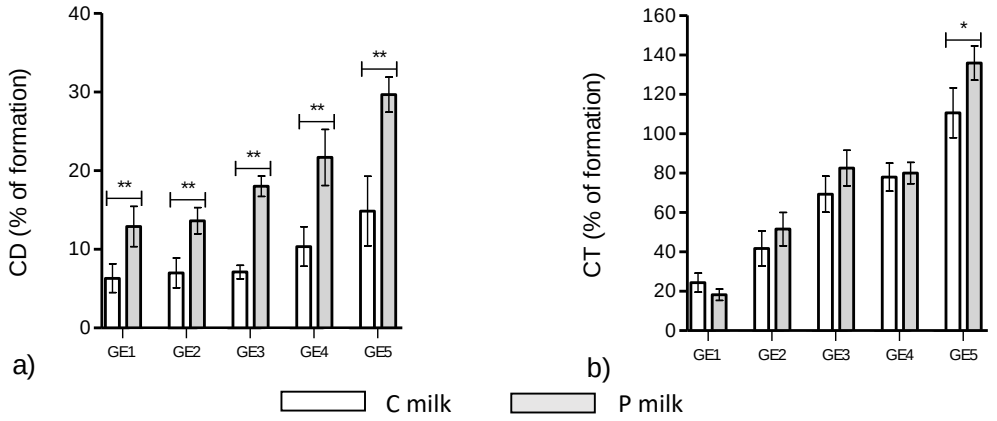

Figure 2.2. Formation of primary oxidation products (a) conjugated dienes (CD) and (b) trienes (CT) during gastric digestion in a semi-dynamic *in vitro* model. Each data point is the average, and error bars represent the standard deviation of two experiments (two replicates per gastric emptying, GE, in each experiment). **P* < 0.05, ***P* < 0.01.

In the present study, a vessel with a lid with openings was used, as suggested in the selected method. The unused openings were covered with a plastic film, and the opening for removing GE samples was also covered with a plastic film between samplings. CD are the first relatively stable chemical marker of oxidation in PUFA, and CT are formed by simultaneous oxidation of higher PUFA and further oxidation of CD monohydroperoxides; both are primary oxidation products and arise in early stages of lipid oxidation.[34] No decrease in the CD and CT formation during semi-dynamic IVD (GE1-GE5) was observed, possibly indicating that no considerable further oxidation occurs to yield secondary products of oxidation. P milk is potentially more prone to oxidation because it contains more PUFA, which justifies the higher percentage of formation of CD when compared with C milk (Figure 2a). On the other hand, an identical (*P* > 0.05) percentage of formation of CT was observed (Figure 2.2.b) for both milks in all GE points, except for GE5 where a slight increase was

noticed. The similar percentage of CT formation for both milks in GE1/2/3/4 could be due to the relative stability of CD, leading to a gradual progression of the oxidative reaction. The role of antioxidants on the oxidative stability of the lipids in milk appears to be less important than the variation in the FA profile. Indeed, Smet et al.[16] studied the oxidative stability during storage of two nutritionally different UHT milks, a milk enriched with unsaturated FA against a non-enriched (control), and observed that, after depletion of the antioxidants in the serum phase, the formation of primary oxidation products increased more rapidly for the milk higher in unsaturated FA than for the control milk, reaching a plateau sooner. Moreover, in the present study, the total antioxidant capacity in the selected milks was similar; this indicates that the differences observed in the formation of lipid oxidation products was related to differences in the FA composition between the C milk and the P milk.

2.3.3. Lipolysis during semi-dynamic gastric digestion

The quantification of FFA in the mimicked stomach was possible using the INFOGEST semi-dynamic method due to gradual acidification and inclusion of gastric lipase. When Van Hekken et al.[6] simulated the digestion of milk from cows on pasture and on conventional management using a static IVD method, no lipolysis occurred during the gastric phase because it was performed without inclusion of gastric lipase. Regarding the pH (Figure 2.3), which is a key factor in gastric lipase activity, a similar pH was observed between milks in all GE points ($P > 0.05$), starting with a pH value of ~4.90 in GE1 for both milks and reaching a value below 2.0 at the end of the gastric IVD. No significant differences ($P > 0.05$) were observed on the percentage of total FFA for the C milk and the P milk in each GE point (Figure 2.3). The relative percentage of total FA increased considerably up to GE2, then leveled off in the GE3 point, and decreased in the two last GE points ($P > 0.05$) (Figure 2.3). Accordingly, homogenized UHT whole milks present high release of FA in the early points of gastric digestion.[19] This is caused by the particular consistency of the coagulum that is formed in the *in vitro* stomach. Heat treatment of milk induces an association between whey proteins and *κ*-casein, leading to the formation of a very soft coagulum, which is more available to gastric enzymes and hydrochloric acid.[35] Moreover, gastric lipase presents optimum hydrolysis activity between pH 4 and 6. The cumulative relative release of total FA at the simulated gastric phase was about 5% for both milks; this value is in accordance with the literature that describes a hydrolysis of 5−40% of dietary fat in the stomach.[36] The formation of CD and CT during gastric IVD could have negatively influenced the level of free PUFA in GE points, lowering the cumulative release of total FA.

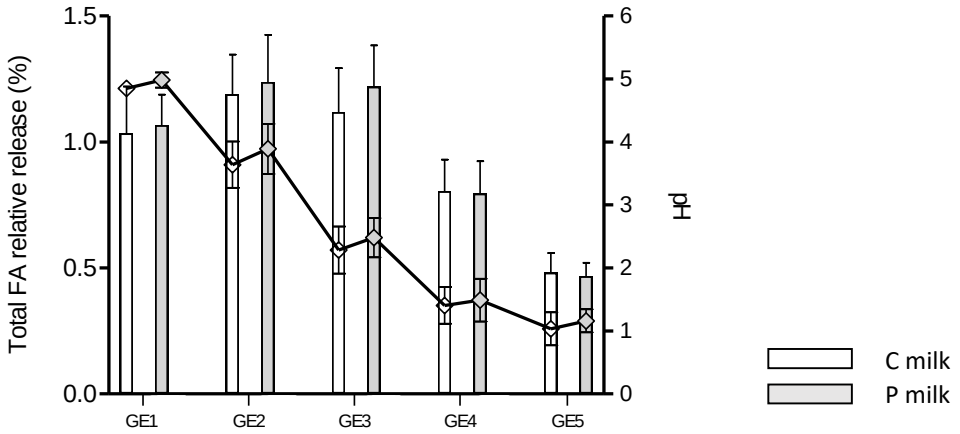

Figure 2.3. Relative release of total fatty acids (FA) expressed as the percentage of the corresponding total FA mass in the ester form present in the undigested milks (columns) and pH values (lines) of the conventional (C) milk and the pasture-based (P) milk during gastric digestion in a semi-dynamic *in vitro* model. Each data point is the average, and error bars represent the standard deviation of two experiments (two replicates per gastric emptying, GE, in each experiment). $P > 0.05$.

Results of relative release of individual FA are presented in Figure 2.4. The cumulative relative release of C8:0 (~16%), C10:0 (~20%), and C12:0 (~11%) was on average higher than those of C14:0 (~2.5%), C16:0 (~2.5%), and C18:0 (~3.5%) (longer chain FFA), which agrees with the chain-size FA and positional specificity of gastric lipase.[7] Although milk FA up to C12:0 are mainly esterified at the *sn*-3 position, milk PUFA prefer primary (*sn*-1,3) positions.[37] The proportion of some PUFA at the *sn*-3 position in milk TAG is influenced by milk FA composition and therefore, the comparison of relative release of PUFA between the selected milks at the gastric phase was also evaluated despite their susceptibility to oxidative degradation. Over gastric IVD (GE1-GE5), the relative release of FA differed ($P < 0.05$) between milks regarding C8:0, C18:2n-6, C18:3n-3, and CLA*c*9*t*11 (Figure 2.4).

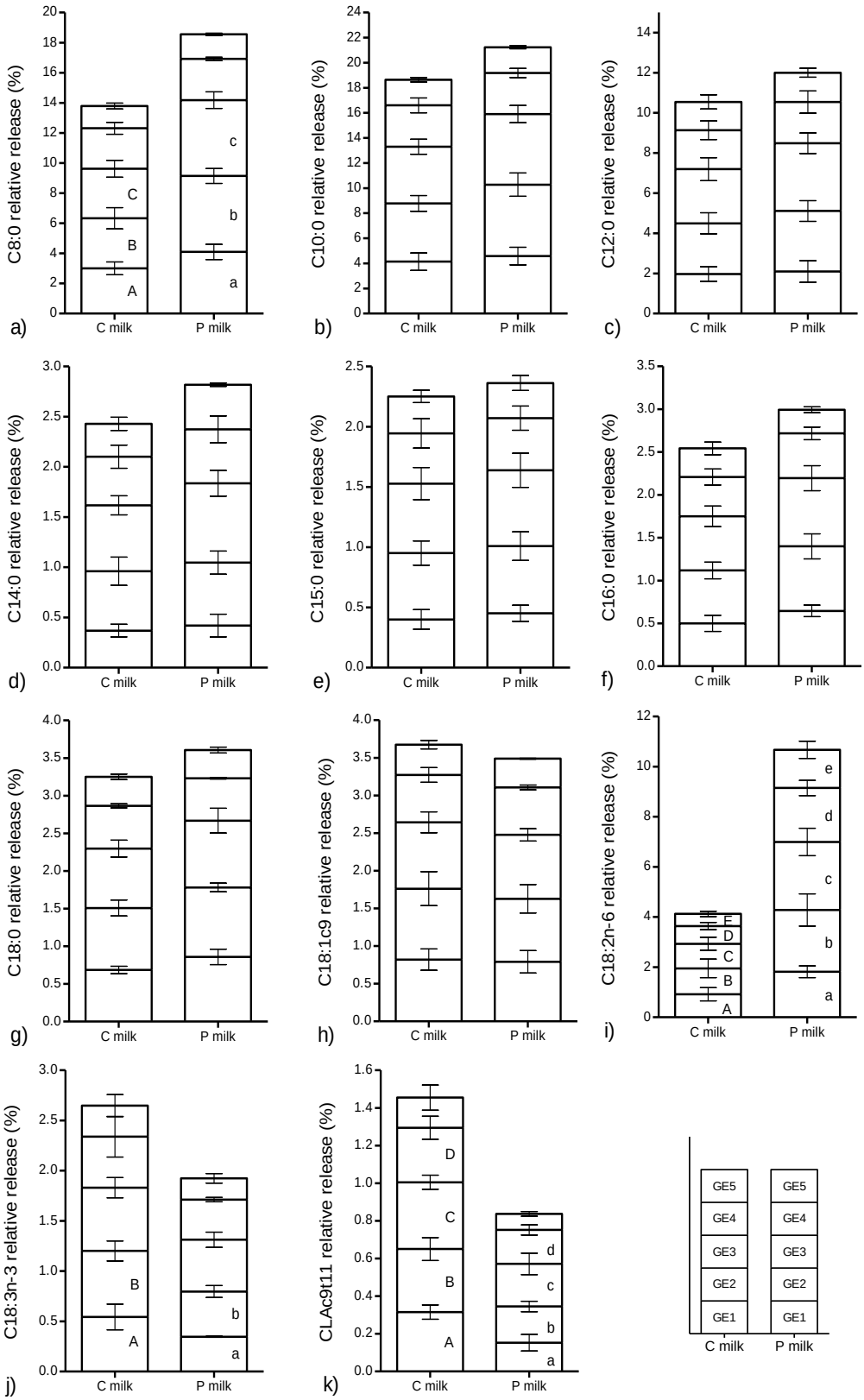

Figure 2.4. (a−k) Relative release of individual fatty acids (FA) of selected milks during gastric digestion in a semi-dynamic *in vitro* model expressed as the percentage of the corresponding FA mass in the ester form present in the undigested milks. Each data point is the average, and error

bars represent the standard deviation of two experiments (two replicates per gastric emptying, GE, in each experiment). *P < 0.05, **P < 0.01, ***P < 0.001.

Specifically, the P milk had a higher relative percentage of C8:0 in GE1/2/3 and a higher relative percentage of C18:2n-6 in all GE points. The C milk had a higher relative percentage of C18:3n-3 in GE1/2 and a higher relative percentage of CLA-*c*9*t*11 in GE1/2/3/4. Accordingly, Tzompa-Sosa et al.[10] reported that a milk with lower proportion of C16:0, such as the P milk, had a higher amount of C8:0 at the *sn*-3 position. Concerning C18:2n-6, C18:3n-3, and CLA-*c*9*t*11, a recent review by Cossignani et al.[37] highlighted that there is a lack in the literature regarding the complete stereospecific determination of long-chain FA in TAG of blended/commercial dairy samples. For instance, Valeille and Martin[9] reported difficulties in obtaining a reliable positional determination of CLA isomers in TAG of Comté-cheese fats ripened for different times. Therefore, alongside with the relative release of FA, their absolute quantification is also a valuable data. Specifically, the quantity of free C18:2n-6, C18:3n-3, and CLA*c*9*t*11 at the gastric phase was higher for the P milk than for the C milk (P < 0.001, Figure 2.5) in spite of the lower proportion of C18:2n-6 (P < 0.05, Table 2.1) and the lower relative release of C18:3n-3 (GE1/2) and CLA*c*9*t*11 (GE1/2/3/4) (Figure 2.4).

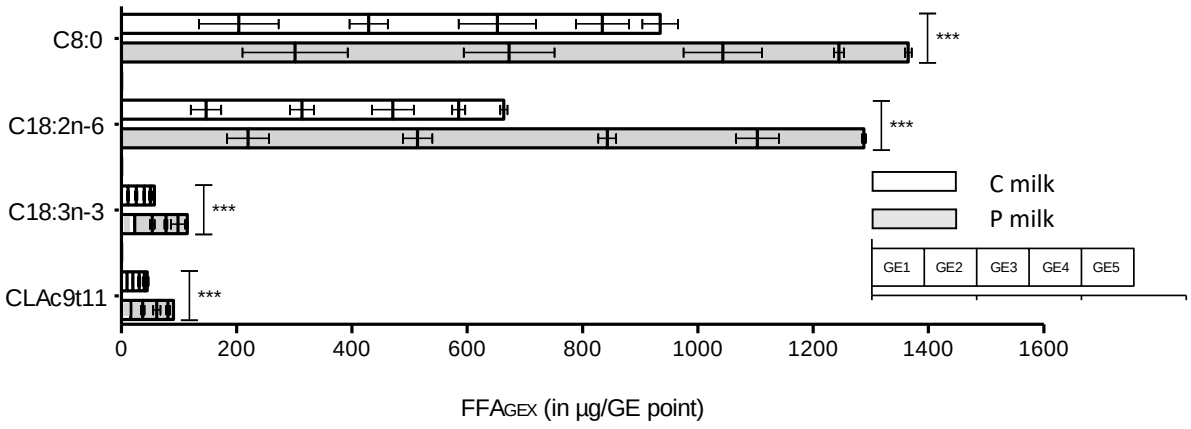

Figure 2.5. Free C8:0, C18:2n-6, C18:3n-3, and CLA*c*9*t*11 mass (in μg) at the gastric emptying (GE) points (FFA_{GEx}) for the conventional (C) milk and the pasture-based (P) milk. Each data point is the average, and error bars represent the standard deviation of two experiments (two replicates per GE point in each experiment). ***P < 0.001.

2.3.4. Free FA and potential effects on metabolism

Quantitatively, the P milk provided 35% more of free C8:0 at the gastric phase compared to the C milk (P < 0.001, Figure 2.5). The FA up to C12:0 are absorbed directly into the portal circulation and transported to the liver for rapid β-oxidation, thus being readily used as a

source of energy and resulting in faster satiety.[38] The P milk provided twice the amount of free C18:2n-6 and 75% more of free C18:3n-3 and free CLA*c*9*t*11 than the C milk at the gastric phase. The FA with a chain length of >12C are effective releasers of the cholecystokinin, a hormone recognized to suppress appetite, and therefore of paramount importance for healthy weight management.[39] In addition, unsaturated long-chain FFA rapidly activate the pancreatic lipase−colipase complex, which is required for the efficient lipolysis process in the intestine, avoiding steatorrhea and consequent fluid losses and deficiencies of fat-soluble vitamins.[36,40] Pancreatic lipase−colipase is responsible for the major lipolysis in the intestine, having activity toward the FA in the primary (*sn*-1,3) positions of the di- and triacylglycerols. The FA in the *sn*-2 positions are subjected to a non-enzymatic isomerization to 1-monoacylglycerols under the alkaline conditions of the small intestine, which makes them available for enzymatic hydrolysis, potentially allowing full TAG hydrolysis.[41] To simulate the intestinal digestion of food, the semi-dynamic *in vitro* model of digestion applied in the present work suggests performing the INFOGEST static version of the intestinal phase for each aliquot emptied from the gastric phase. Nonetheless, the static models do not reproduce the dynamic environment of the intestine, such as peristaltic movements and continuous changes in pH and secretion flow rates.[42] For instance, the INFOGEST static version of the intestinal phase recommends a constant pH of 7, while the pH varies from about 7 to between 6 and 5 in the duodenum due to acidic gastric emptying.[2] Therefore, the present study was focused only on the gastric lipolysis and lipid oxidation of commercial cow's milks with different FA profiles, applying the semi-dynamic *in vitro* model of INFOGEST, which is a model that provides physiologically relevant data regarding the gastric phase of digestion, including kinetic aspects such as gradual acidification and fluid and enzyme secretion and emptying.[2]

In conclusion, differences in gastric behavior of milks with different FA compositions were successfully determined by the percentage of CD and CT formation, the relative release of total and individual FA (expressed as percentage of the corresponding FA mass in the ester form present in the undigested milks), and the absolute quantification of individual FFA after gastric digestion. This may influence the absorption rate of FFA in the intestine and subsequently induce different human metabolic responses.

2.4. Funding

This work was supported by FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operational Programme for Competitiveness and Internationalisation (POCI) and by Portuguese funds through FCT - Fundação para a

Ciência e a Tecnologia in the framework of the project PTDC/SAU-NUT/30322/2017. The work was supported through the project UIDB/50006/2020 funded by FCT/MCTES through national funds.

Notes

The authors declare no competing financial interest.

2.5. Acknowledgments

S.C.M.P. thanks Fundação para a Ciência e a Tecnologia, SANFEED Doctoral Programme, and SORGAL (Sociedade de Óleos e Rações S.A., S. João Ovar, Portugal) for her Ph.D. grant (PD/BDE/135539/2018). M.F. acknowledges FCT/MEC - Fundação para a Ciência e Tecnologia, Ministério da Educação e Ciência for his researcher contract.

2.6. Abbreviations used

TAG, triacylglycerols; FA, fatty acids; C, carbons; IVD, *in vitro* digestion; PUFA, polyunsaturated fatty acids; UHT, ultra-heat treatment; C milk, conventional milk; P milk, pasture-based milk; RGE, rabbit gastric extract; FAME, fatty acid methyl ester; ORAC, oxygen radical absorbance capacity; CD, conjugated dienes; CT, conjugated trienes; SSF, simulated salivary fluid; eSSF, simulated salivary electrolyte fluid; SGF, simulated gastric fluid; eSGF, simulated gastric electrolyte fluid; GE, gastric emptying; FFA, free fatty acids; n, omega; *c*, *cis*; *t*, *trans*; CLA, conjugated linoleic acid

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CHAPTER 3 – Gastric epithelial response to milk fat using the semi-dynamic INFOGEST digestion model coupled with NCI-N87 cells

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Gastric epithelial response to milk fat using the semi-dynamic INFOGEST digestion model coupled with NCI-N87 cells

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Submitted

Abstract

The stomach is a relevant spot of lipolysis for milk fat, but research on the effect of digested milk fat in the gastric epithelium is scarce and difficult to evaluate. In the present study, we implemented the semi-dynamic *in vitro* digestion model of INFOGEST, combined with gastric NCI-N87 cells, to study the effect of fat-free, whole conventional, and whole pasture-based milk on gastric epithelium. Cellular messenger ribonucleic acid (mRNA) expression of membrane fatty acids receptors (GPR41, GPR84), antioxidant enzymes (CAT, SOD, GPX), and inflammatory molecules (NF-κB p65, IL-1β, IL-6, IL-8 and TNF-α) was assessed. No significant differences were observed in mRNA expression of GPR41, GPR84, SOD, GPX, IL-6, IL-8, and TNF-α, after exposure of the NCI-N87 cells to milk digesta samples ($p > 0.05$). An increase of CAT mRNA expression was observed ($p < 0.05$), at a similar level, for all milk types. Whole milk digested samples induced higher mRNA expression of NF-κB p65 and IL-1β than fat-free milk ($p < 0.05$); while no differences were observed between whole conventional and whole pasture-based milk ($p > 0.05$). Moreover, the effect of milk digesta on gastric mRNA expression was studied in a scenario of subsequent stimulation of NCI-N87 monolayer with the pro-inflammatory cytokine IFN-γ. In these conditions, milk digesta samples increased CAT mRNA expression ($p < 0.05$), but had no effect in the expression of NF-κB p65 and IL-1β ($p > 0.05$). The increase of CAT mRNA expression suggests that milk fatty acids are used for energy production by gastric epithelial cells. Cellular antioxidant response to higher milk fatty acids availability could be associated to gastric epithelial inflammation, but did not contribute to increased inflammation in case of an external insult with IFN-γ. Besides, a conventional or a pasture-based origin did not affect the impact of whole milk in the NCI-N87 monolayer. The combined model responded to differences in milk fat content, which indicates its usefulness to study effects of foods at the gastric level.

Keywords: conventional milk, pasture-based milk, gastric models, interferon-γ, quantitative PCR

3.1. Introduction

Milk is an important dietary source of essential vitamins and minerals, high-quality proteins, and fats (Comerford et al., 2021). The relationship between milk fat and health has been widely investigated in humans, but some results are inconsistent (Zhang et al., 2021). In recent years, a topic of great interest in the field of food science is the understanding of the outcome of food digesta in the human digestive system, because gastrointestinal epithelium is the first tissue that interacts with nutrients released from food (Giromini et al., 2019; Sensoy, 2021). The interaction of milk digesta with the gastric epithelium is thus of great importance in providing tools towards a better understanding of the effects of milk on health. The stomach is a relevant spot of lipolysis and metabolism for milk fat. Indeed, milk fat is rich in short- (~4% w/w) and medium-chain fatty acids (8-12% w/w; SCFA and MCFA), which are predominantly located at the *sn*-3 position and gastric lipase exhibits preference for cleavage at the *sn*-3 position and specificity to SCFA and MCFA (Bauer et al., 2005). The SCFA can be absorbed by gastric cells through diffusion and carrier-mediated anionic exchange (Saunders, 1991; Bugaut and Bentéjac, 1993). Moreover, gastric cells express membrane free fatty acids (FFA) receptors and a long-term intake of dietary fat is supposed to be associated with up-regulation of membrane FFA receptors in the stomach (Widmayer et al., 2015; Nunez-Salces et al., 2020).

Fatty acids can be involved in cell signaling processes, which affect gene expression and health (Calder, 2015). In metabolically active organs (e.g. liver, heart, and brain), FFA can regulate pathways essentially intertwining metabolism and inflammation through FFA receptor-mediated signaling (Miyamoto et al., 2016; Kimura et al., 2020). Moreover, intermediate metabolites generated in FFA catabolism for cellular energy production participate in several important physiological processes, such as inflammation (Rindler et al., 2013; Carthew, 2021).

To study the interplay between food and gut cells, *in vitro* digestion methods coupled with cell culture models have been increasingly used as they provide a good representation of *in vivo* gastrointestinal conditions, involving fewer ethical restrictions and allowing high-throughput studies in controlled conditions (Giromini et al., 2019). Although several gastric cancer cell lines are available (e.g. AGS, KATO-III, MKN-28 and NCI-N87 cells), only NCI-N87 cells exhibit the capacity to form physiologically relevant tight and coherent monolayers (Basque et al., 2001). In addition, *in vitro* models allow the simulation of different gastric luminal conditions enabling a better understand of cellular responses induced by foods. Indeed, very different conditions can be found in the human stomach as increased concentrations of pro-inflammatory cytokines (e.g., IFN-γ, IL-1β, TNF-α) due to the direct contact of the epithelium with noxious substances as alcohol or pathogens (e.g.,

Helicobacter pylori and Epstein - Barr virus) (Nie and Yuan, 2020). Specifically, IFN-γ is a key pro-inflammatory mediator; its receptor is expressed on all types of nucleated cells and IFN-γ induces multiple cellular responses, such as enhancing the production of reactive oxygen species (ROS) (Kak et al., 2018).

The goal of this work was to study the effect of milks differing in fat content and fatty acids profile in gastric epithelial mRNA expression of membrane fatty acids receptors (GRP41 and GPR84), antioxidant enzymes (CAT, SOD, GPX), NF-κB p65, and pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α). For that purpose, we selected the INFOGEST semi-dynamic digestion model, described by Mulet-Cabero (2020), to simulate milk digestion and obtain physiologically mimetic digestas further exposed to NCI-N87 epithelium. In detail, selected milk samples (fat-free, whole conventional, and whole pasture-based milk), after semi-dynamic *in vitro* digestion, were repeatedly applied to the NCI-N87 monolayer over 5 days simulating regular consumption. The mRNA expression of referred genes was assessed after: i) exposition to digested milk samples and ii) subsequent inflammatory stimulation with IFN-γ at a concentration that enhances intracellular ROS production. This work is pioneer in associating the semi-dynamic INFOGEST model of digestion with a cellular model of gastric epithelium to study the gastric epithelial response to milk, following the physiological dynamics of stomach digestion of a complex food as milk.

3.2. Material and methods

3.2.1. Milk samples

Homogenized UHT whole conventional milk (i.e., no management label), whole pasture-based milk, and fat-free milk (premium brand) were obtained from retail outlets in November 2020. As soon as milk samples were purchased, they were transferred to 50 mL sterile plastic tubes (Sarstedt, Nümbrecht, Germany) and stored at -80 °C. Selected whole milk samples showed differences in their fatty acid profile and gastric lipolysis extent as reported by Pinho et al. (2021a).

3.2.2. Reagents and cells

Rabbit gastric extract (RGE15) was purchased from Lipolytech (Marseille, France). Pepsin (P7012), calcium chloride dehydrate, 2',7'-dichlorofluorescindiacetate (DCFH-DA), phosphate buffered saline tablet (PBS), IFN-γ human recombinant expressed in *E. coli*, bovine serum albumin (BSA), 1-methoxyphenazine methosulfate (MPMS), lithium L-lactate, β-nicotinamide adenine dinucleotide sodium salt (NAD), iodonitrotetrazolium chloride (INT), 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), and RNase-free water were from

Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was obtained from a Milli-Q gradient system (18.2 mΩ/cm) from Millipore (Milford, DE, USA). Roswell Park Memorial Institute (RPMI) 1640 Medium and Dulbecco's modified Eagle's medium (DMEM) were purchased from Lonza (Basel, Switzerland). Fetal bovine serum (FBS), 0.25% trypsin EDTA solution, penicillin/streptomycin 100× solution (penicillin, 10,000 U/mL; streptomycin, 10,000 mg/mL) were purchased from Gibco/Life Technology (Paisley, UK). PureZOL RNA isolation reagent, iScript cDNA synthesis kit, iTaq universal SYBR green supermix were purchased from Bio-Rad (Hercules, CA, USA); GelRed nucleic acid stain was from Biotium (Fremont, CA, USA). Primers sequences used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were synthesized by Eurofins Genomics (Ebersberg, Germany). The human gastric epithelial cell line NCI-N87 was kindly provided by Institute for Research and Innovation in Health (Porto, Portugal).

3.2.3. In vitro *semi-dynamic gastric digestion*

Human gastric digestion was simulated following the standardized semi-dynamic protocol described by Mulet-Cabero et al. (2020). A 15 mL sample was used and detailed parameters applied for simulated oral and gastric digestion steps are summarized in Table 3.1. Gastric emptying (GE) was performed in five points, at a linear rate, using a pipette with an end internal diameter of 2 mm. Before each GE, chyme was mixed to collect a fraction representative of the whole gastric content at that time point. The pH of each GE was recorded and immediately increased to 7.0±0.2 with 2 M NaOH to inactivate pepsin (pH meter BASIC 20^{+}, Crison, Barcelona, Spain). Then, GE samples were centrifuged at 5,000 ×*g* for 30 min at 4 °C twice (Centrifuge 5810R, Eppendorf, Enfield, CT, USA) to eliminate solid residues (Xia et al., 2017). A second oral digestion was performed to obtain a sample of the oral phase. Aliquots of oral sample and each GE were stored at -80 °C until analysis. A blank of digestion consisting of 15 mL of water was treated with SSF, SGF, and enzymes mixture at the same concentration and rate determined for the digestion of milk samples. Samples and blank digested in duplicate and further stored as pooled samples.

Immediately before the cell treatments, oral phase and the five GE digestas were defrosted at room temperature and mixed to mimic the dynamic average exposure of gastric cells, as stomach emptying occurs, following the scheme presented in Figure 3.1A. Specifically, five combined digestas (in a ratio of 1:1, v/v) were prepared, namely: mix digesta 1 (oral phase sample and GE1); mix digesta 2 (GE1 and GE2); mix digesta 3 (GE2 and GE3); mix digesta 4 (GE3 and GE4); and mix digesta 5 (GE4 and GE5). Then, digesta mixes were diluted 12 times in DMEM; this dilution was previously used by Rafiee Tari et al. (2019) and Pinho et

al. (2021b) for cell assays, with *in vitro* digested dairy foods, to ensure no toxicity of the digests in the cell monolayer.

Table 3.1. Oral and gastric conditions for the INFOGEST *in vitro* semi-dynamic digestion.

		Fat-free milk[1]	**Whole milk[2]**
Oral digestion step			
Quantity of food (mL)		15	15
Energy content of meal (Kcal)		5.04	9.78
Simulated salivary fluid	Electrolyte simulated salivary fluid (mL)	1.008	1.416
	0.3 M $CaCl_2(H_2O)_2$ (µL)	6.3	8.9
	Water (mL)	0.246	0.346
Gastric digestion step			
Simulated gastric fluid (90%)[3]	Electrolyte simulated gastric fluid (mL)	11.382	11.739
	0.3 M $CaCl_2(H_2O)_2$ (µL)	8.1	8.4
	1.5 M HCl (mL)[4]	1.460	1.460
Enzyme solution (90%)[3]	Water (mL)	1.784	1.886
	RGE solution (mL)[5]	0.813	0.839
	Pepsin solution (mL)[6]	0.813	0.839
Simulated gastric fluid rate (mL/min)		0.2306	0.2381
Enzyme solution rate (mL/min)		0.026	0.027
Number of emptying steps		5	5
Emptying volume (mL)[7]		6.50	6.71
Emptying time, every (min)[7]		11.42	11.41
Total digestion time (min)[7]		57.12	57.05

[1]0% fat, 3.4% protein, and 5.0% carbohydrates (macronutrient content described on nutritional labeling). [2]Whole conventional and whole pasture-based milks = 3.6% fat, 3.3% protein, and 4.9% carbohydrates (macronutrient content described on nutritional labeling). [3]The other 10% were previously added into the reaction vessel. [4]Volume and concentration of HCl required to decrease the pH of milk to pH 2. [5]Lipase activity = 12 units/mg and pepsin activity = 220 units/mg. [6]Pepsin activity = 2145 units/mg. [7]Gastric emptying dynamics were calculated based on the delivery of 2 kcal/min of a milk volume of 340 mL of fat-free milk and 175 mL of whole fat milk based on *in vivo* considerations, according to Mulet-Cabero et al. (2020).

3.2.4. Cell assays

3.2.4.1. Cell culture

NCI-N87 cell line was grown in 75 cm^2 culture flasks at 37 °C in a 5% CO_2 humidified atmosphere in complete medium, which was constituted by RPMI-1640 with 10% heat inactivated FBS and 1% penicillin/streptomycin. At 80-90% confluence, cells were trypsinized and seeded at 5.5 × 10^5 cells/cm^2 in 96-well plates for lactate dehydrogenase (LDH) and reactive oxygen species (ROS assays); or at 2.5 × 10^5 cells/cm^2 in 12-well plates (Sarstedt) for the mRNA expression analysis. Cells were cultured during a period of 8 (96-well plate) or 18 days (12-well plate) in complete medium renewed every 2 days until the beginning of assays and daily thereafter. All experiments were carried out between cell passages 16 to 38.

3.2.4.1.2. Lactate dehydrogenase (LDH) release and intracellular reactive oxygen species (ROS) production

The response of the NCI-N87 monolayer to the pro-inflammatory cytokine IFN-γ was determined in a preliminary test. The cytoplasmic LDH released into the cell culture medium was determined to assess IFN-γ-induced cytotoxicity. On the 8th day *post*-seeding in 96 well plates, cells were incubated with IFN-γ from 0 to 100 ng/mL (diluted in DMEM) for 4 h; then, LDH levels were determined in the supernatant by absorbance at 490 nm in a microplate reader (BioTek, Winooski, VT, USA) following the protocol described by Kaja et al. (2015). The results were expressed as a percentage of the absorbance of the DMEM without IFN-γ treatment, which was set to 100%. Two independent experiments with six replicates per treatment were performed.

Intracellular ROS production was measured by incubating cells in a 96-well plate with DCFH-DA, as a fluorescence probe (Wang et al., 2018). In detail, cells on the 8th day *post*-seeding were first incubated in PBS with 10 mM DCFH-DA for 30 min at 37 °C in a 5% CO_2 humidified atmosphere and then supernatant was replaced by IFN-γ from 0 to 30 ng/mL (diluted in DMEM) for 4 h; lastly, the supernatant was replaced by PBS and ROS levels were immediately determined by fluorescence intensity (Exc: 488 nm; Em: 525 nm). The results were expressed as a percentage of the fluorescence value for the DMEM without IFN-γ treatment. Two independent experiments with six replicates per treatment were performed.

3.2.4.2. Cell treatments

The NCI-N87 monolayer in 12-well plate was repeatedly treated with blank of digestion, fat-free milk, whole conventional milk, or whole pasture-based milk samples (after semi-

dynamic *in vitro* digestion) over 5 days, mimicking long-term intake. Chosen exposure duration was recommended by the European Centre for the Validation of Alternative Methods (Pfaller et al., 2001). Experimental design is schematically presented in Figure 3.1B. In detail, on the 1st day of treatment, cells were washed twice with warm PBS and then exposed to digesta mix 1 for 30 min at 37 °C in a 5% CO_2 humidified atmosphere. After this period, digesta mix 1 was carefully aspirated, substituted by digesta mix 2, and then incubated for further 30 min. Cells were further exposed to mixes 3, 4 and 5 following the same substitution procedure. Finally, after 30 min exposure to mix 5, cells were washed twice with warm PBS and incubated in complete medium for 22 h. Cells were equally subjected to this procedure on the 2nd, 3rd, 4th, and 5th day of treatment (Figure 3.1B). On the 5th day of treatment, RNA extractions were carried out immediately after: i) exposition to digested milk samples from semi-dynamic *in vitro* gastric digestion and ii) exposition to digested milk samples and subsequent inflammatory stimulation with 30 ng/mL IFN-γ for 4h (Figure 3.1C). To evaluate mRNA expression levels, a reference for each condition was included. The reference consisted of cells treated with DMEM instead of *in vitro* digested samples over 5 days (normal condition) along with a single exposure to IFN-γ in the last day (IFN-γ-stimulated condition). Cell treatments started on the day 14th *post*-seeding and finished on the day 18th *post*-seeding. Cells were exposed to digesta treatments or DMEM for a total of 2.5 h per day. This daily experimental duration mimicked the time needed for gastric digestion of a 300 kcal breakfast meal. Two independent experiments with two replicates per sample were performed, in four biological replicates per sample.

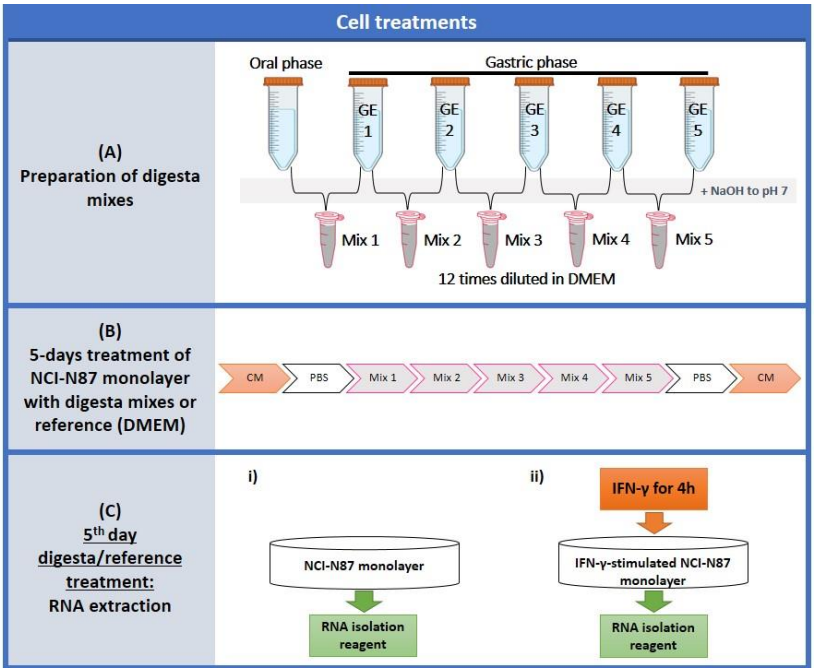

Figure 3.1. Schematic representation of the (A) preparation of digesta mixes, the (B) treatment of NCI-N87 monolayer with digesta mixes, and the (C) RNA extraction after, i) exposition to digesta

mixes and ii) subsequent inflammatory stimulation with IFN-γ : GE – gastric emptying, DMEM – Dulbecco's modified Eagle's medium, CM – complete medium, PBS – phosphate buffered saline tablet.

3.2.4.3. mRNA expression analysis

Total RNA of the NCI-N87 monolayer was extracted with PureZOL RNA isolation reagent, according to the manufacturer's protocol. RNA was quantified (Gen5 software from BioTek plate reader, USA) from UV absorbance at 260 and 280 nm (Take3 adapter). The integrity of RNA was assessed by electrophoresis on a denaturing agarose gel stained with GelRed (Biotium, Fremont, USA), visualized under a UV light tray (Gel Doc EZ System, Bio-Rad). Then, 1.0 µg RNA was reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad) in 20 µL reactions; cDNA was stored at -25 °C until analysis. The qPCR reaction mixture was prepared in duplicate and each replicate consisted of cDNA diluted 20 times with nuclease free water (1 µL), iTaq Universal SYBR Green Supermix (5 µL), RNase-free water (3.2 µL) and primer sets at 10 µM (2× 0.4 µL). Non template controls without cDNA were included in each reaction. Amplification was performed in a real-time PCR system (CFX Connect, Bio-Rad) with the following conditions: one denaturation step at 95 °C for 40 s followed by 40 cycles at 95 °C for 15 s and annealing at 60 °C for 60 s. Amplification specificity was confirmed by melting curve analysis (65 to 95 °C, increment 0.5 °C). The expression level of GPR41, GPR84, CAT, SOD, GPX, NF-κB p65, IL-1β, IL-6, IL-8, and TNF-α was calculated by the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). Graphs are presented in $\log_2$ of $2^{-\Delta\Delta Cq}$ values for better visualization of underexpressed values. The list of primer sequences is presented in Table 3.2. Treated samples (blank of digestion, fat-free milk, whole conventional milk, or whole pasture-based milk samples) were expressed against reference (DMEM or DMEM with 30 ng/mL IFN-γ) and normalized to the expression of the housekeeping genes ACTB and GAPDH. The mRNA expression data graphs were prepared using GraphPad v. 7.05 (GraphPad Software, La Jolla, CA, USA).

Table 3.2. Sequences of the primers used for reverse transcription-qPCR.

Gene	Primer	Sequence (5' to 3')
ACTB	Forward	CACTCTTCCAGCCTTCCTTCC
	Reverse	GCACTGTGTTGGCGTACAGG
GAPDH	Forward	CAACGGATTTGGTCGTATTGG
	Reverse	GCAACAATATCCACTTTACCAGAGTTAA
GPR41	Forward	ACCTGCTGGCCCTGGTG
	Reverse	GGTCAGGTTGAGCAGGAGCA
GPR84	Forward	TTCAGCCCTTCTCTGTGGACA
	Reverse	TGCAGAAGGTGGCACCG
CAT	Forward	GCAGATACCTGTGAACTGTC
	Reverse	GTAGAATGTCCGCACCTGAG
SOD	Forward	GCACATTAACGCGCAGATCA
	Reverse	AGCCTCCAGCAACTCTCCTT
GPX	Forward	CCTCAAGTACGTCCGACCTG
	Reverse	CAATGTCGTTGCGGCACACC
NF-kB p65	Forward	ATGTGGAGATCATTGAGCAGC
	Reverse	CCTGGTCCTGTGTAGCCATT
IL-1β	Forward	ATGATGGCTTATTACAGTGGCAA
	Reverse	GTCGGAGATTCGTAGCT
IL-6	Forward	TCCACAAGCGCCTTCGGTCCAG
	Reverse	CTCAGGGCTGAGATGCCGTCG
IL-8	Forward	ATGACTTCCAAGCTGGCCGTGGC
	Reverse	TCTCAGCCCTCTTCAAAAACTTCTC
TNF-α	Forward	CTGCTGCACTTTGGAGTGAT
	Reverse	AGATGATCTGACTGCCTGGG

3.2.5. Statistical analysis

The statistical analysis of the results was performed using GraphPad v. 7.05. Each data point is the average of two independent experiments, and error bars represent the standard error of the mean. The differences between treatments were assessed by one-way ANOVA followed by Tukey's *post hoc* test. For all comparisons, the level of significance was determined at a $p < 0.05$.

3.3. Results

3.3.1. IFN-γ-induced production of reactive oxygen species (ROS) at non cytotoxic levels

The concentrations 0, 10, 20, 30, 40, 50, and 100 ng/mL IFN-γ were applied to the NCI-N87 monolayer resulting in an increase on LDH levels in the cell culture medium at the concentrations of 40, 50, and 100 ng/mL compared to the DMEM without IFN-γ stimulus ($p < 0.05$; Figure 3.2).

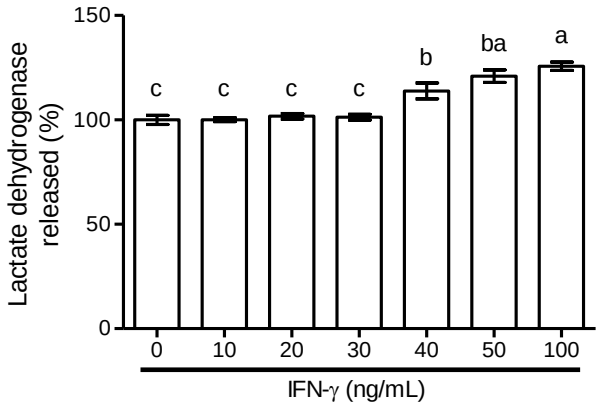

Figure 3.2. Levels of lactate dehydrogenase (LDH) in the culture medium of NCI-N87 monolayer intestinal exposed to different IFN-γ concentrations (0, 10, 20, 30, 40, 50 and 100 ng/mL). Each data point is the mean of two independent experiments, and error bars represent the standard error of the mean. The level of significance was determined at $p < 0.05$.

Additionally, the intracellular ROS levels were assessed in the NCI-N87 monolayer exposed to above determined non-cytotoxic concentrations of IFN-γ (i.e., 0, 10, 20, and 30 ng/mL). Results indicate that only the concentration 30 ng/mL IFN-γ was able to enhance significantly intracellular ROS production compared to DMEM without IFN-γ treatment ($p < 0.05$; Figure 3.3). To further study the role of *in vitro* digested milks in the IFN-γ-stimulated NCI-N87 monolayer, the concentration 30 ng/mL IFN-γ was thus selected, ensuring enhanced intracellular ROS production without cell death. Higher ROS production is central to the progression of inflammation (Kak et al., 2018).

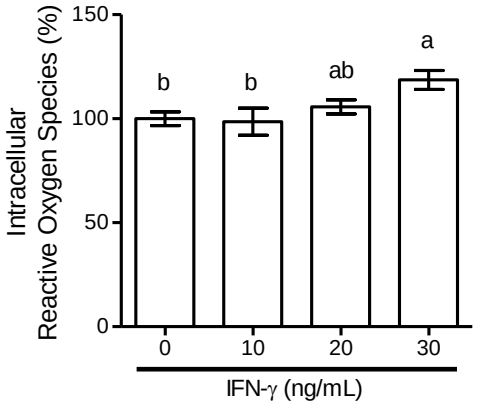

Figure 3.3. Intracellular reactive oxygen species (ROS) levels on the NCI-N87 monolayer exposed to non-cytotoxic concentrations of IFN-γ (0, 10, 20, and 30 ng/mL). Each data point is the mean of two independent experiments, and error bars represent the standard error of the mean. The level of significance was determined at $p < 0.05$.

3.3.2. mRNA expression of free fatty acids receptors GPR41 and GPR84

The blank of digestion (absence of milk), fat-free, whole conventional, and whole pasture-based milks presented similar GPR41 mRNA expression in the NCI-N87 monolayer, as well as in the IFN-γ-stimulated NCI-N87 monolayer ($p > 0.05$; Figure 3.4 A,B). In relation to GPR84 mRNA expression, digesta treatments had similar values in both simulated gastric luminal conditions ($p > 0.05$; Figure 3.4 C,D), presenting the same behavior as GPR41 expression.

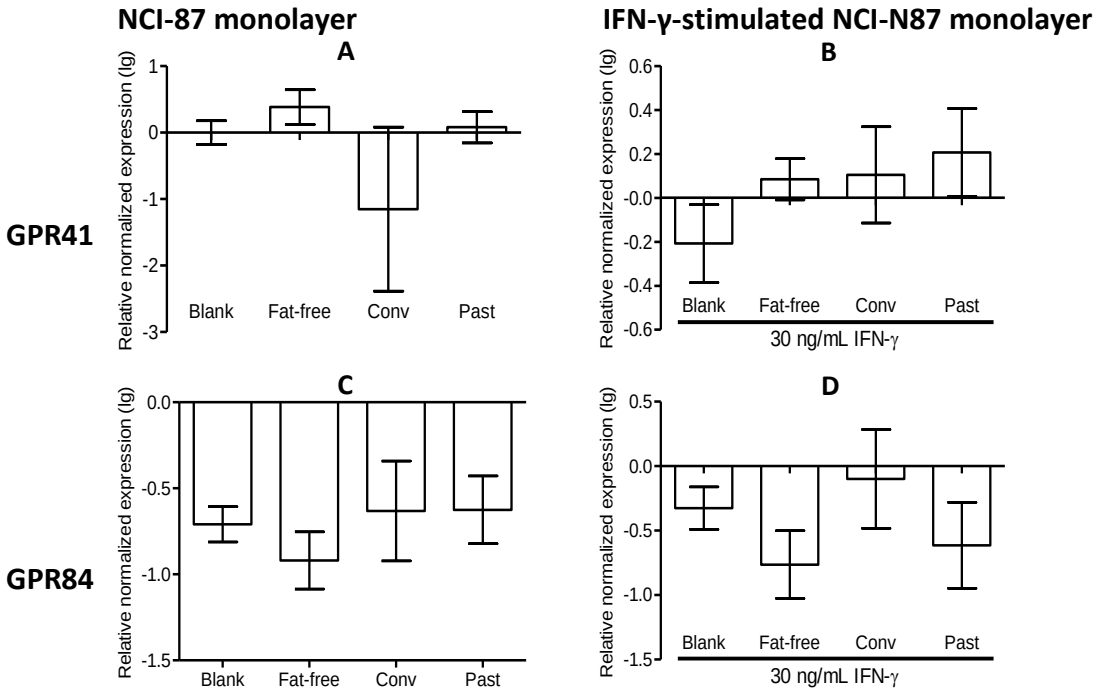

Figure 3.4. (A-D) Effect of digesta treatments on GPR41 and GPR84 mRNA expression in the NCI-N87 monolayer: Blank – blank of digestion, Fat-free – *in vitro* digested fat-free milk, Conv – *in vitro* digested whole conventional milk, Past – *in vitro* digested whole pasture-based milk. Relative normalized expression was calculated based on the $2^{-\Delta\Delta Cq}$ method. ACTB and GAPDH were used as housekeeping genes. The reference in the NCI-N87 monolayer was cells treated with DMEM instead digesta, whereas the reference in the IFN-γ-stimulated NCI-N87 monolayer was cells exposed to DMEM with IFN-γ at 30 ng/mL. Each data point is the mean of two independent experiments, and error bars represent the standard error of the mean. The level of significance was determined at $p < 0.05$.

3.3.3. mRNA expression of antioxidant enzymes CAT, SOD, and GPX

The fat-free and whole milks induced higher CAT expression than the blank of digestion in both simulated gastric luminal states – normal and IFN-γ-stimulated ($p < 0.05$). No significant differences in CAT mRNA expression were observed among the fat-free, whole conventional, and whole pasture-based milks ($p > 0.05$, Figure 3.5 A,B). Regarding SOD and GPX, digesta treatments induced similar expression values in the NCI-N87 cells, as well as in the IFN-γ-stimulated treatment ($p > 0.05$; Figure 3.5 C-F).

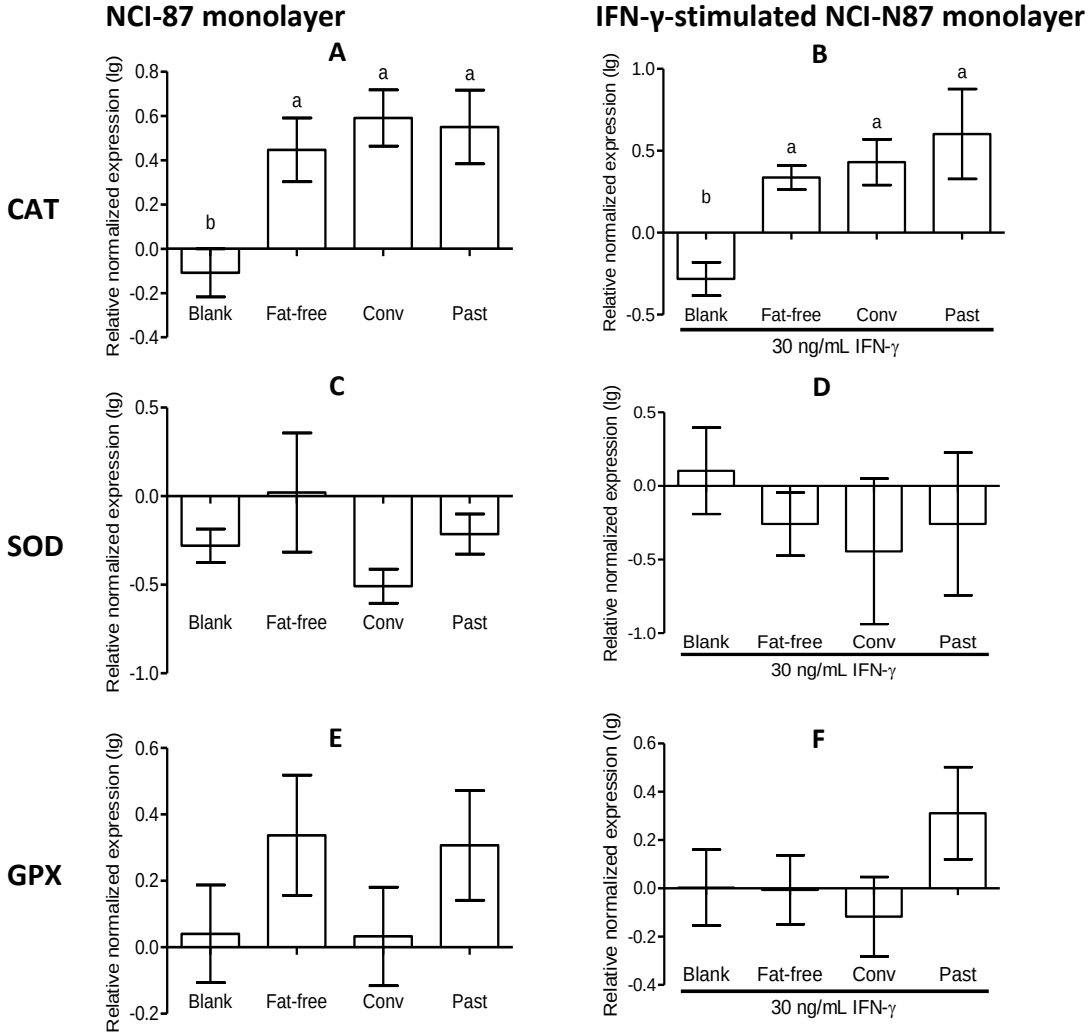

Figure 3.5. (A-F) Effect of digesta treatments on CAT, SOD, and GPX mRNA expression in the NCI-N87 monolayer: Blank – blank of digestion, Fat-free – *in vitro* digested fat-free milk, Conv – *in vitro* digested whole conventional milk, Past - *in vitro* digested whole pasture-based milk. Relative normalized expression was calculated based on the $2^{-\Delta\Delta Cq}$ method. ACTB and GAPDH were used as housekeeping genes. The reference in the NCI-N87 monolayer was cells treated with DMEM instead digesta, whereas the reference in the IFN-γ NCI-N87 monolayer was cells exposed to DMEM with IFN-γ at 30 ng/mL. Each data point is the mean of two independent experiments, and error bars represent the standard error of the mean. The level of significance was determined at $p < 0.05$.

3.3.4. mRNA expression of NF-κB p65, IL-1β, IL-6, IL-8, and TNF-α

Digesta treatments presented differences on NF-κB p65 and IL-1β mRNA expression ($p < 0.05$; Figure 3.6 A,C) after exposition to NCI-N87 gastric epithelium. Nonetheless, in the IFN-γ-stimulated monolayer, digesta treatments presented similar NF-κB p65 and IL-1β expression ($p > 0.05$; Figure 3.6 B,D). In relation to IL-6, IL-8, and TNF-α, digesta treatments showed similar values in both the non-stimulated and the IFN-γ-stimulated monolayers ($p > 0.05$; Figure 3.6 E-J). Specifically, whole milks induced higher mRNA expression of NF-κB p65 and IL-1β than the fat-free milk ($p < 0.05$); and no differences were observed between whole conventional and whole pasture-based milk treatments ($p > 0.05$; Figure 3.6 A,C). Additionally, mRNA level of IL-1β was increased by the fat-free milk compared to the blank of digestion, in which IL-1β appears down regulated ($p < 0.05$; Figure 3.6C).

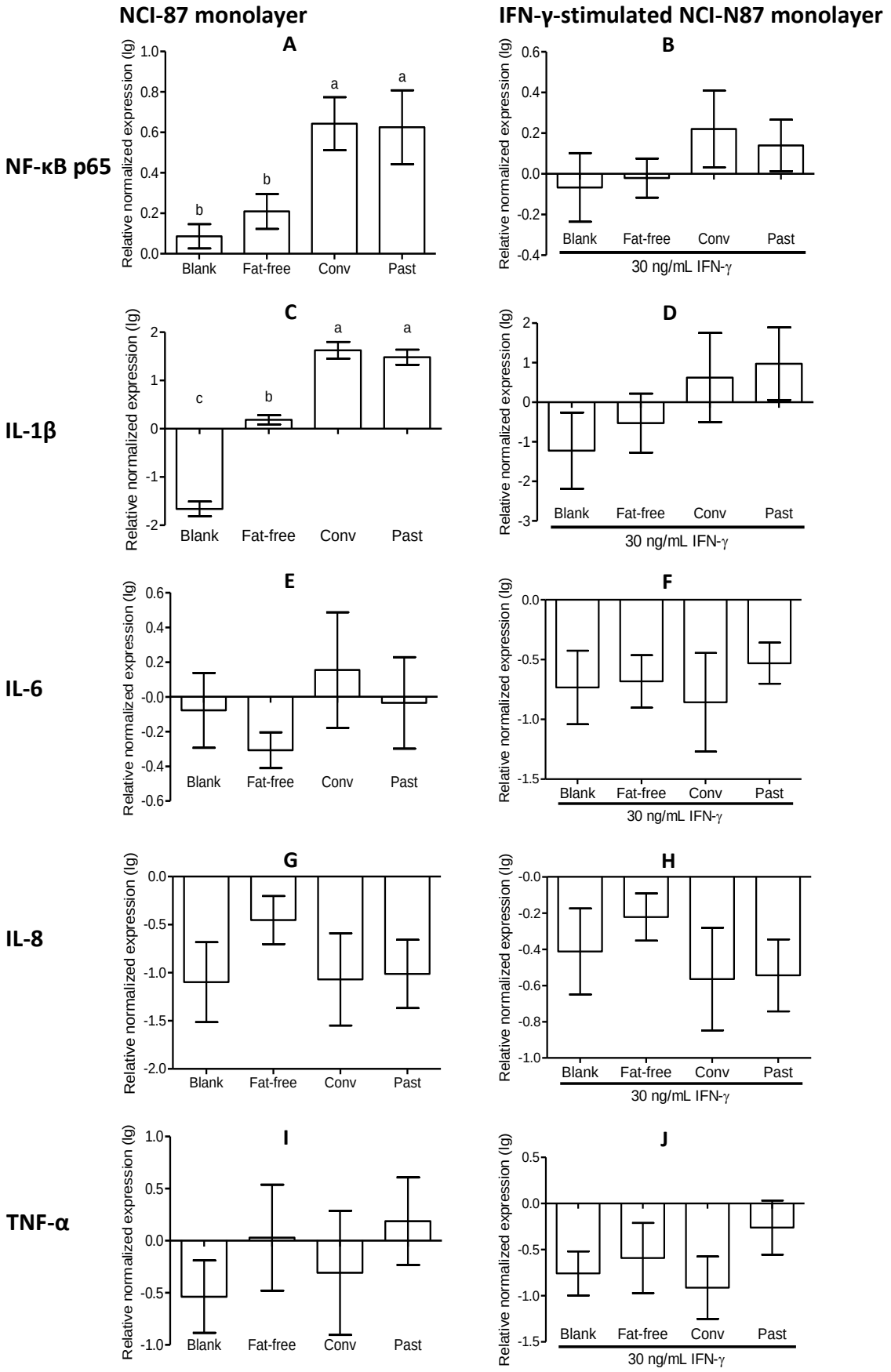
NCI-87 monolayer
IFN-γ-stimulated NCI-N87 monolayer
NF-κB p65
IL-1β
IL-6
IL-8
TNF-α
A
B
C
D
E
F
G
H
I
J
Relative normalized expression (lg)
Blank
Fat-free
Conv
Past
30 ng/mL IFN-γ

Figure 3.6. (A-J) Effect of digesta treatments on NF-κB p65, IL-1β, IL-6, IL-8, and TNF-α in the IFN-γ-stimulated NCI-N87 monolayer: Blank – blank of digestion, Fat-free – *in vitro* digested fat-free milk, Conv – *in vitro* digested whole conventional milk, Past – *in vitro* digested whole pasture-based milk. Relative normalized expression was calculated based on the $2^{-\Delta\Delta Cq}$ method. ACTB and GAPDH were used as housekeeping genes. The reference in the NCI-N87 monolayer was cells treated with DMEM instead digesta, whereas the reference in the IFN-γ-stimulated NCI-N87 monolayer was cells exposed to DMEM with IFN-γ at 30 ng/mL. Each data point is the mean of two independent experiments, and error bars represent the standard error of the mean. The level of significance was determined at $p < 0.05$.

3.4. Discussion

In the present study, the INFOGEST standardized semi-dynamic *in vitro* digestion model, which dynamic pH profile and emptying times were based on data from studies *in vivo*, combined with gastric epithelial NCI-N87 cells exposed to digestas obtained, was used to study the effect of fat-free, whole conventional, and whole pasture-based milk on gastric cellular mRNA expression. The effect of milk digestas in the expression of membrane fatty acid receptors GPR41 (G protein-coupled receptor responding to SCFA) and GPR84 (G protein-coupled receptor responding to MCFA) were firstly assessed. A high-fat diet is supposed to be associated with changes in the expression level of membrane FFA receptors in the stomach (Widmayer et al., 2015; Nunez-Salces et al., 2020), but no differences on the expression of GPR41 and GPR84 were observed between treatments in the present study. In line with our results, Widmayer et al. (2015) and Nunez-Salces et al. (2020) observed that the intake of dietary fat had no effect on the relative mRNA amounts of SCFA receptors in the stomach of mice fed with a high-fat diet compared to mice fed with a standard chow diet. Widmayer et al. (2015) also determined the number of epithelial and enteroendocrine cells, reporting an increase in the number of the latter on the gastric mucosa of mice fed with a high-fat diet compared to mice fed with a standard chow diet. Accordingly to the cited authors, a high-fat diet seems not to induce changes in the expression level of membrane FFA receptors, which was confirmed in the *in vitro* dynamic physiomimetic condition used herein, but rather changes in the cellular repertoire.

On the other hand, high fat diets were shown to up-regulate the H_2O_2-metabolizing enzyme catalase, which constitutes a specific response to accommodate the increased oxidation of FFA for energy production while not perturbing H_2O_2-mediated signaling (Rindler et al., 2013). Likewise, we observed, in both simulated gastric luminal conditions, an increase of CAT mRNA expression for the whole conventional and the whole pasture-based milks compared with the blank of digestion. Moreover, whole milks had a similar effect in CAT

compared with the fat-free milk. This could be due to the gradual gastric emptying of FFA (simulated in the semi-dynamic digestion and mimicked in the cell treatments), which leads to the accumulation of small amounts of FFA released from the whole conventional and the whole pasture-based milks in the gastric lumen. Moreover, the blank of digestion lacks the amino acids from milk digesta; amino acids are used by cells as precursors for metabolically active proteins (Liu et al. 2017), leading to different cellular responses between the blank of digestion and the fat-free milk.

A high-fat diet contributes to inflammation and the NF-κB family of transcription factors are key regulators in transcription of pro-inflammatory genes (Renaud et al., 2014). Up to this point of the discussion, it was seen that pre-treatment with the whole milks led to higher mRNA expression of the H_2O_2-metabolizing enzyme CAT. Since H_2O_2 has been implicated in the regulation of the NF-κB family of transcription factors (Mitchell and Carmody, 2018), it is expected that an increase in CAT could lead to an increase in these factors. In fact, in the NCI-N87 monolayer, it was observed a rise of the NF-κB p65 and IL-1β mRNA expression for the whole conventional and the whole pasture-based milks compared with the blank of digestion. Besides, whole milks caused higher NF-κB p65 and IL-1β expression than the fat-free milk, which supports that the cellular uptake of SCFA and their metabolism for energy production (with generation of H_2O_2) had occurred in the NCI-N87 monolayer. Therefore, cellular metabolism of whole milks contributes to the up-regulation of pro-inflammatory NF-κB p65 and IL-1β in the non-stimulated NCI-N87 monolayer.

The up-regulation of inflammatory molecules by whole milk samples draw attention to the effect of milk samples with subsequent IFN-γ-stimulation of the NCI-N87 monolayer. During disease states, the production of cytokines is higher than during health, which can lead to functional consequences within the epithelium (Stadnyk, 1994). For instance, IL-1β causes an increase in epithelial tight junction permeability in an NF-κB dependent manner (Al-Sadi et al. 2010). Since milk samples increased CAT expression in the IFN-γ-stimulated NCI-N87 monolayer, it could be expected the up-regulation of pro-inflammatory NF-κB p65 and IL-1β. However, it was observed that the intermediate metabolites generated in the metabolism of fatty acids did not cause the up-regulation of the NF-κB p65 and IL-1β in the IFN-γ-stimulated NCI-N87 monolayer. Therefore, the present results suggest that milk nutrients availability did not contribute to increased epithelial inflammation in an environment of higher pro-inflammatory cytokines (mimicked in the IFN-γ-stimulated NCI-N87 monolayer).

The pro-inflammatory cytokines IL-6, IL-8, and TNF-α are also important regulatory signals in cellular response to environmental modifications, but no significant differences in their mRNA expression were observed among digesta treatments. It has been reported that in complex environments some cytokines are not present, because there is a cross talk, which

establishes a hierarchy of cytokine responsiveness (Chu et al., 2013). In the present study, cells were under multiple complex cellular environments (availability of different kind of nutrients in both normal and inflammatory states, mimicking the physiological situations after a meal ingestion), which indicate the importance of environment-related hierarchies in responsiveness.

3.5. Conclusions

In the present study, the *in vivo* validated semi-dynamic in vitro digestion model combined with gastric epithelial NCI-N87 cells exposed to digestas was used to study the gastric epithelial response to fat-free, whole conventional, and whole pasture-based milk digestion at the mRNA expression level. Results showed that milk nutrients availability induced important molecular responses in the simulated gastric epithelium. Indeed, *in vitro* digested fat-free and whole milks increased mRNA expression of H_2O_2-metabolizing enzyme catalase. Cellular antioxidant response to higher milk fatty acids availability could be associated to gastric epithelial inflammation, but did not contribute to increased inflammation in the scenario of an external insult with IFN-γ at non-cytotoxic levels. Besides, a conventional or a pasture-based origin of the fat in the whole milks did not influence the effect in the NCI-N87 monolayer. The combined implementation of the semi-dynamic *in vitro* digestion model with matching sequential epithelial exposure to digestas responded to differences in milk fat content, which indicates the model usefulness for high-throughput studies related to health effects of complex foods at the gastric level.

Conflicts of interest

Authors declare no conflicts of interest.

3.6. Funding

This work was supported by Portuguese national funds (FCT/MCTES, Fundação para a Ciência e Tecnologia and Ministério da Ciência, Tecnologia e Ensino Superior) through the project UIDB/50006/2020 and by the project DIETxPOSOME (PTDC/SAU-NUT/6061/2020). Susana C.M. Pinho was supported by SANFEED Doctoral Programme, FCT, and SORGAL (Sociedade de Óleos e Rações S.A., S. João Ovar, Portugal) (Ph.D. grants PD/BDE/135539/2018 and COVID/BDE/152525/2022). Miguel A. Faria acknowledges FCT/MEC – Fundação para a Ciência e Tecnologia, Ministério da Educação

e Ciência – the researcher contract. This study was supported by the European Union through FEDER funds (NORTE-01-0145-FEDER-000052).

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CHAPTER 4 – Effect of skimmed milk on intestinal tract: Prevention of increased reactive oxygen species and nitric oxide formation

International Dairy Journal 118 (2021) 105046

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Effect of skimmed milk on intestinal tract: Prevention of increased reactive oxygen species and nitric oxide formation

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Effect of skimmed milk on intestinal tract: Prevention of increased reactive oxygen species and nitric oxide formation

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Cite this: Pinho, S. C. M., Faria, M. A., Melo, A., Pinto, E., Almeida A., Alves, R., et al. (2021). Effect of skimmed milk on intestinal tract: Prevention of increased reactive oxygen species and nitric oxide formation. *International Dairy Journal,* 118, 105046, https://doi.org/10.1016/j.idairyj.2021.105046

Abstract

The capacity of skimmed milk to neutralise increased reactive oxygen species (ROS) and to attenuate nitric oxide (NO) production, as well as to present cytoprotective effect at the intestinal level was assessed after *in vitro* gastro-intestinal digestion. The impact on ROS modulation was evaluated at a non-cytotoxic concentration of hydrogen peroxide (H_2O_2) in a co-culture of Caco-2 and HT-29 intestinal cells. In parallel, a cytotoxic concentration of H_2O_2 was used to study the effect of digested milk against induced cell apoptosis. Concerning induced NO production, it was evaluated using the model lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells. Results showed that digested milk prevented the increase of basal ROS level in the intestinal epithelium and attenuated NO production by LPS-stimulated macrophage cells. In the H_2O_2-induced cytotoxicity assay, digested milk had no protection against apoptosis, confirmed by the failure in attenuating activated caspase-3/7.

Keywords: skimmed milk, amino acids, minerals, intestinal cell model, human health

4.1. Introduction

Reactive oxygen species (ROS) and nitric oxide (NO) are physiologically produced during cellular metabolism (Forrester, Kikuchi, Hernandes, Xu, & Griendling, 2018; Mu, Yu, & Kitts, 2019). However, an overproduction of ROS with depletion of the antioxidant system causes

cell damage or death if the injury cannot be repaired (Forrester et al., 2018). In addition, uncontrolled production of NO by inducible nitric oxide synthase (iNOS) propagates cell damage, due to its reaction with ROS, which produces highly toxic reactive nitrogen species (Mu et al., 2019).

In particular, the gastrointestinal tract (GI) is constantly exposed to multiple external triggers that stimulate ROS generation, such as alcohol, heavy metals ions, and *trans* fatty acids (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014). When the intestinal antioxidant system is overwhelmed ROS accumulation occurs, which damages intestinal epithelial cells (Circu & Aw, 2012). Upon injury, epithelial cells release inflammatory mediators, leading to chemotaxis and activation of polymorphonuclear neutrophils and monocytes/macrophages that express iNOS, producing large amounts of NO. This results in further exacerbation of epithelial injury and the reduction of intestinal barrier function (Chokshi, Hunter, Guner, Grishin, & Ford, 2008; Wang, Ye, Zeng, & Qiao, 2019). Loss of barrier function increases intestinal permeability, which is currently believed to be responsible for the pathogenesis of intestinal (e.g., inflammatory bowel disease, irritable bowel syndrome, celiac disease) and systemic diseases (e.g., multiple sclerosis, non-alcoholic fatty liver disease, asthma), as it facilitates the passage of bacteria, toxins, and allergens to sub-epithelial lamina propria or enter the portal circulation (Camilleri et al., 2019; Vancamelbeke & Vermeire, 2017). Therefore, modulating ROS formation and iNOS activation are important underlying mechanisms to ensure intestinal protection against several stimuli promoting an overall physiological wellbeing (Mu et al., 2019).

Milk is a nutrient-rich food, supplying high-quality protein and a range of essential micronutrients (Gorska-Warsewicz, Rejman, Laskowski, & Czeczotko, 2019; Shilpashree, Arora, Kapila, & Sharma, 2018). Moreover, milk proteins are recognised as one of the main sources of biologically active peptides (Boutrou, Henry, & Sanchez-Rivera, 2015; Ferreira et al., 2007). These peptides can be defined as specific amino acids (AA) sequences that have physiological significance beyond the pure nutritional requirements and they may ultimately influence health (Kitts & Weiler, 2003).

In recent years, some studies have been published considering the effect of individual milk peptides obtained by simulated GI digestion from caseins or whey proteins in counteracting higher ROS level in intestinal Caco-2 monolayers exposed to hydrogen peroxide (H_2O_2) (García-Nebot, Cilla, Alegría, & Barberá, 2011; Laparra, Alegría, Barberá, & Farré, 2008), as well as induced NO production by stimulated RAW264.7 macrophage cells (Bamdad, Bark, Kwon, Suh, & Sunwoo, 2017). Overall, these studies demonstrate that individual milk-derived peptides may neutralise ROS or attenuate NO production. However, some authors have highlighted that since foods do not consist of single nutrients, linking one food-derived

compound to a health effect is an important finding, but unrealistic (Cilla, Alegría, Barberá, & Lagarda, 2013; Thorning et al., 2017); other compounds are also involved in physiological radical reactions, such as free AA, released from proteins (Egger et al., 2019; Tagliazucchi, Helal, Verzelloni, & Conte, 2016), or other milk constituents, such as minerals. Further research on the potential health effects of dairy products alongside the more traditional approach of studying the health effects of single nutrients is thus required (Giromini, Cheli, Rebucci, & Baldi, 2019). *In vitro* assays are useful tools to study ROS modulation at the intestine level using the H_2O_2-stimulated Caco2/HT-29 model of intestinal epithelium (Ferraretto et al., 2018), whereas NO production by iNOS can be assayed using the LPS-stimulated RAW264.7 macrophage model (Bamdad et al., 2017).

The present study aims to evaluate the potential of skimmed milk after GI digestion neutralise high level of ROS and attenuate NO production, as well as its potential cytoprotective effect, at the intestinal tract using *in vitro* assays.

4.2. Materials and methods

4.2.1. Samples

To cover the main dairy regions and milk players in the Portuguese market, two commercial brands of milk from the Portuguese mainland and two from the Azores Islands were purchased. For each brand, 3 milk packages were acquired in June 2019 with a similar "best before" date, from different grocery stores and further analysed as a pooled sample.

4.2.2. Reagents, standards, and cell lines

Ortho-phthaldialdehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC), iodoacetic acid, phenol, sodium azide (NaN_3), pancreatin (P7545), bile extract (B8631), pepsin (P6887), calcium chloride dihydrate ($CaCl_2(H_2O)_2$), trichloroacetic acid (TCA), 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 2',7'-dichlorofluorescindiacetate (DCFH-DA), phosphate buffered saline tablet (PBS), LPS from Escherichia coli, sulphanilamide, and N-(naphth-1-yl) ethylenediamine dihydrochloride were from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydrogen phosphate (Na_2HPO_4) and sodium tetraborate decahydrate ($Na_2B_4O_7.10H_2O$) were purchased from Merck (Darmstadt, Germany). Sodium hydroxide tablet (NaOH) was from LabChem (Zelienople, PA, USA).

Methanol and acetonitrile of HPLC grade, ortho-phosphoric acid 85% (H_3PO_4) and hydrochloric acid 32% (HCl) were from Merck.

Tetramethylammonium hydroxide solution (TMAH) was from Sigma-Aldrich. Nitric acid (HNO_3) for trace metal analysis (69% w/w, Trace Select) was from Fluka (St. Gallen, Switzerland). Hydrogen peroxide (H_2O_2) for trace metal analysis (30-32%, w/w, Primar) was purchased from Fisher Chemical (Loughborough, UK). H_2O_2 for cell assays (30% w/w, containing stabiliser) was from Sigma-Aldrich. Ultrapure water purified by Milli-Q gradient system (18.2 mΩ/cm) from Millipore (Milford, DE, USA).

Dulbecco's modified Eagle's medium (DMEM), minimum essential medium non-essential amino acids 100 × , GlutaMAX 100 × , foetal bovine serum (FBS), 0.25% trypsin-EDTA solution, penicillin/streptomycin 100 × solution (penicillin, 10,000 U mL^{-1}; streptomycin, 10,000 mg mL^{-1}) were purchased from Gibco/Life Technology (Paisley, UK). CellEven caspase-3/7 green detection reagent was purchased from Invitrogen (Waltham, MA, USA). AA standards and norvaline (internal standard for AA analysis) were from Sigma-Aldrich. ERM-BD151 skimmed milk powder (certified reference material for trace elements) was supplied by the European Commission's Science (Geel, Belgium).

The human epithelial cell line Caco-2 was provided by the “Molecular Physical-Chemistry” group of the University of Coimbra, while the human-derived epithelial cells HT-29 were purchased from Sigma-Aldrich. The macrophage-like cell line derived from BALB/c mice, RAW264.7, was kindly provided by the “Laboratory of Pharmacognosy” of the Faculty of Pharmacy, University of Porto.

4.2.3. Characterisation of samples

4.2.3.1. Amino acid profile

The amino acid (AA) composition of each milk sample was determined after sample hydrolysis with 6 M HCl e phenol solution at 110 °C for 24 h in presence of 0.5% phenol and norvaline for all AA except Trp, according to the reference hydrolysis method (AOAC 982.30; AOAC, 1990a). For Cys analysis, before the acidic hydrolysis, samples were diluted in a reducing solution, stored under nitrogen during 2 h at room temperature in the dark, and then an iodoacetic acid solution was added for an additional 30 min at room temperature, according to the method 9 of the European Pharmacopoeia (EP, 2005). For Trp analysis, samples were hydrolysed with 4.2 M NaOH for 18 h at 110 °C (AOAC 988.15; AOAC, 1990b). After cooling to 4 °C, the hydrolysates from acidic hydrolysis were neutralised with NaOH, whereas hydrolysates from alkaline hydrolysis were neutralised with HCl. Neutralised hydrolysates were diluted with 0.1 M borate buffer and centrifuged at 4000×*g* for 5 min. Supernatants were filtered through a 0.22 µm syringe filter followed by analysis by RP-HPLC/fluorescence detection, with pre-column OPA/FMOC derivatisation (Marino et al., 2010).

An analytical HPLC unit (Jasco, Tokyo, Japan) equipped with an FP-920 fluorescence detector was used. The column used was a Zorbax Eclipse Plus C18, 4.6 × 250 mm i.d. column, prepacked with 5 µm particles (Agilent, Santa Clara, CA, USA). The mobile phase A was 10 mM Na_2HPO_4 with 10 mM $Na_2B_4O_7$, pH adjusted to 8.2, and 5 mM NaN_3. The mobile phase B was acetonitrile: methanol: ultrapure water (45:45:10, vol: vol: vol). Three analytical replicates were run for each milk sample. Individual AA peaks were identified by comparing with the retention times of the corresponding peaks obtained in the calibration standards. AA profile was expressed as percentage of the total AA (m/m).

4.2.3.2. Mineral content

Milk samples were mineralised with HNO_3 and H_2O_2 in a closed vessel microwave digestion system for all minerals except I (Pacquette et al., 2018). For I determination, samples were prepared using TMAH extraction at high temperature (EN 15111; EN, 2007). The minerals Ca, Mg, K, and Na were analysed by flame atomic absorption spectrophotometry using an AAnalyst 200 flame (air-acetylene) atomic absorption spectrometer (PerkinElmer, Überlingen, Germany). The minerals P, Mn, Fe, Cu, I, Zn, Se, Mo, Cr, and Co were analysed by inductively coupled plasma-mass spectrometry with an iCAP Q (Thermo Fisher Scientific, Schwerte, Germany) instrument. To check the accuracy of the analytical procedures, a skimmed milk powder certified reference material was used. Three analytical replicates for each milk sample were run and blank values were run alongside samples and subtracted from the sample readings before the results were calculated. The contents of individual minerals were expressed per 100 g of milk.

4.2.3.3. Fat content

Fat content was determined as a control measure of skimmed milk according to the method ISO 2446 (ISO, 2008).

4.2.4. In vitro *gastrointestinal digestion*

Immediately after the acquisition, milk samples were subjected to *in vitro* digestion. Human simulated GI digestion was performed according to the internationally harmonised static protocol described by Minekus et al. (2014). Briefly, 5 mL milk was mixed with 4.5 mL simulated salivary fluid without amylase, 25 µL 0.3 mM $CaCl_2$ and 475 µL ultrapure water. After 2 min of incubation, the oral content was mixed with 8 mL of simulated gastric fluid, 500 µL pepsin (2000 U mL^{-1}), 5 µL 0.3 mM $CaCl_2$, 425 µL 1 M HCl and 970 µL ultrapure water. The gastric mixture was immediately incubated for 2 h and then mixed with 8.5 mL of simulated intestinal fluid electrolyte solution, 5 mL pancreatin solution (100 U mL^{-1}), 2.5

mL bile solution (10 mM), 40 µL 0.3 mM $CaCl_2$, 600 µL 1 M NaOH and 3.16 mL ultrapure water (final volume of *in vitro* digesta = 40 mL). The intestinal mixture was immediately incubated for 2 h. All incubations were performed in a water bath at 37 °C with gentle agitation.

After the simulated GI digestion, the enzymes were inactivated by heating at 95 °C for 15 min, as described by Tagliazucchi et al. (2016) followed by immersion in an ice bath. Then, *in vitro* digested milk samples were centrifuged at 5000×*g* for 10 min at 4 °C to separate the bioaccessible fraction and aliquots for the cell assays were stored at -20 °C. Each of the pooled samples was digested in triplicate. A parallel assay was performed to determine the amount of acid or base necessary to adjust the digest pH. An enzyme-blank assay was also included.

Immediately before the cell assays, *in vitro* digested milk was defrosted at room temperature and diluted 12 times in culture medium, which is the dilution previously reported by Rafiee-Tari, Arranz, and Corredig (2019) for cell assays with *in vitro* digested cheese. This dilution also roughly corresponds to 100-fold dilutions of non-digested milk used by Shoji et al. (2005). The enzyme-blank was also diluted 12 times.

4.2.4.1. Free AA profile of *in vitro* digested milk samples

Free AA composition of *in vitro* digested milk samples was analysed after precipitation by TCA (ISO 8968-4; ISO, 2016). TCA-soluble fraction was obtained by mixing equal volumes of *in vitro* digested milk with 24% TCA solution. After 10 min at room temperature, samples were centrifuged at 10,000×*g* for 20 min and the supernatant (12% TCA-soluble fraction) included the free AA. Results were expressed as a percentage of the total free AA (m/m).

4.2.5. Cell assays

4.2.5.1. Cell culture

Caco-2 and HT-29 cells were cultured separately in 75 cm^2 culture flasks and kept at 37 °C in a 5% CO_2 atmosphere, at constant humidity. These cell lines were grown in DMEM medium supplemented with 10% heat-inactivated foetal bovine serum, 1% GlutaMAX solution, 1% nonessential AA solution, and 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin. In the case of RAW264.7 cells, these were grown in 25 cm^2 culture flasks in complete medium constituted by DMEM with 10% heat-inactivated foetal bovine serum and 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin, under 5% CO_2 at 37 °C, in humidified atmosphere. Cell assays were performed under passages 65-81, 13-41, and 8-12, for Caco-2, HT29, and RAW264.7 cells, respectively. Contamination with Mycoplasma was assessed by PCR every three weeks (van Kuppeveld et al., 1992).

4.2.5.2. Caco2/HT-29 co-culture

The *in vitro* model of the human intestinal epithelium, Caco2/HT-29 co-culture (Ferraretto et al., 2018) was used and adapted to a 7-day 96 well-plate, based on the protocol proposed by Galkin, Pakkanen, and Vuorela (2008) for Caco-2 cells. At 80-90% confluence, cells were trypsinised and mixed at the ratio of 7:3, prior to seeding in 96-well plate. Each well was plated with a total of approximately 10^4 cells, which were cultivated during a period of 7 days. Cell confluence was achieved on day 4 and the experiments were performed on the 7^{th} day. Cell confluence was observed under the inverted microscope.

4.2.5.3. Measurement of cell viability and apoptosis

To choose the appropriate concentrations of H_2O_2 to use in the two subsequent assays (induced cytotoxicity protection and prevention of higher ROS level) a preliminary test was conducted. Caco2/HT-29 co-culture was exposed to different concentrations of H_2O_2 (0.2, 0.5, 1.5 and 3.0 mM) during 22 h. Cell viability was assessed using the MTT assay (García-Nebot et al., 2011; Laparra et al., 2008). A concentration that reduced cell viability to about 50% was chosen for the cytotoxicity protection assay and a lower one (promoting an increment in ROS levels without affecting cell viability) was selected to use in the assay concerning the prevention of increased ROS levels.

For the induced cytotoxicity protection assay, cell viability and apoptosis were evaluated. In relation to cell viability measurement, Caco2/HT-29 co-culture was pre-treated with *in vitro* digested milk or complete medium (control) and incubated for 2 h, before the addition of 3 mM H_2O_2, and further incubated for 22 h. After 22 h, the medium was removed and cells were incubated with a 0.5 mg mL^{-1} MTT stock solution during 3 h and then DMSO was added to dissolve the formazan crystals. Absorbance values were measured using a microplate reader (Biotek, Winooski, VT, USA) at 570 nm. The results are expressed as a percentage of the absorbance value of the control (medium only). The control group was set to 100%. Apoptosis was evaluated through the measurement of caspase-3/7 activity (Riss et al., 2013). After 22 h of H_2O_2 exposure following the protocol described above, the medium was removed and 2 µM of CellEvent reagent (diluted in PBS with 5% FBS) was added and further incubated at 37 °C for 30 min. Fluorescence intensity was measured using a microplate reader (Biotek, Winooski, VT, USA) at an excitation wavelength of 502 nm and an emission wavelength of 530 nm. The fluorescence value relative to control (medium only) was set to 100%.

4.2.5.4. Measurement of reactive oxygen species

To study the effect of *in vitro* digested milk on preventing higher ROS level, Caco2/HT-29 co-culture was treated with *in vitro* digested milk or medium and incubated for 2 h, before the addition of 0.2 mM H_2O_2. After 22 h of incubation, ROS were measured intracellularly by incubating cells in PBS containing 10 µM DCFH-DA, as a fluorescence probe at 37 °C (Tonolo et al., 2018). Fluorescence intensity was measured using a microplate reader (Biotek, Winooski, VT, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The results are expressed as a percentage of the fluorescence value for the control group (medium only) which was set to 100%.

4.2.5.5. Measurement of nitrites (nitric oxide)

To evaluate the effect of *in vitro* digested milk on induced NO production, the macrophage-like cell line RAW264.7 was seeded in 96-well plates at a density of 35,000 cells per well, and incubated for 24 h. After this period, the medium was removed, and cells were pre-treated with *in vitro* digested milk or medium only (control). After 2 h, cells were stimulated with 1 µg mL^{-1} of LPS for 22 h (Pereira et al., 2017). NO production was determined by measuring nitrites, a stable end-product of NO metabolism (Calvello et al., 2016). Nitrites were detected in the cell culture medium by mixing 75 mL with an equal volume of Griess reagent [1% sulphanilamide and 0.1% N-(1-naphthyl)ethylenediamine in 2% H_3PO_4]. The plate was incubated for 10 min, in the dark, at room temperature and the absorbance was measured at 560 nm in a microplate reader (Biotek, Winooski, VT, USA). The absorbance values relative to non-pretreated cells stimulated with LPS (positive control) were set to 100% and the results are expressed as relative percentages of the positive control.

4.2.6. Statistical analysis

The statistical analysis of the results was performed using XLSTAT software for Windows version 2016.02 (Addinsoft, Paris, France). The AA profile and mineral composition of milk samples, as well as the free AA profile of *in vitro* digested milk samples, are expressed as mean ± SD of three replicates and the differences between samples means were assessed by one-way ANOVA followed by Tukey's or Tamhane's T2 *post hoc* test, depending on the homogeneity of variances. The results of cell assays are expressed as mean ± SEM of three independent experiments (six replicates per group in each experiment). These results are expressed as a percentage of control and the *post hoc* Dunnett's test was carried out to compare the treatment groups with control group. This comparison test was performed using Prism software, version 7.05 for Windows (GraphPad Software, La Jolla, CA, USA). In addition, the Tukey's *post hoc* test was also carried out for detecting differences between

milk samples means. For all comparisons, a *P*-value lower than 0.05 was considered to denote statistically significant differences.

4.3. Results

4.3.1. Characterisation of milk samples

The four commercial brands of skimmed UHT milk (Mainland 1, Mainland 2, Azores 1, and Azores 2) presented a similar content of total AA ($P > 0.05$) expressed as g 100 g^{-1} (3.18 ± 0.04; 3.20 ± 0.03; 3.16 ± 0.05; 3.17 ± 0.08, respectively for Mainland 1, Mainland 2, Azores 1, and Azores 2). Concerning AA profile, the milk sample Mainland 2 had higher relative content in Phe ($P = 0.04$) and lower relative content in Lys ($P < 0.001$; Figure 4.1A). Regarding the minerals content, milk samples from Mainland had higher content of Cu ($P = 0.001$) and Mo ($P < 0.001$), and lower content of Mn ($P < 0.001$) than milk samples from Azores.

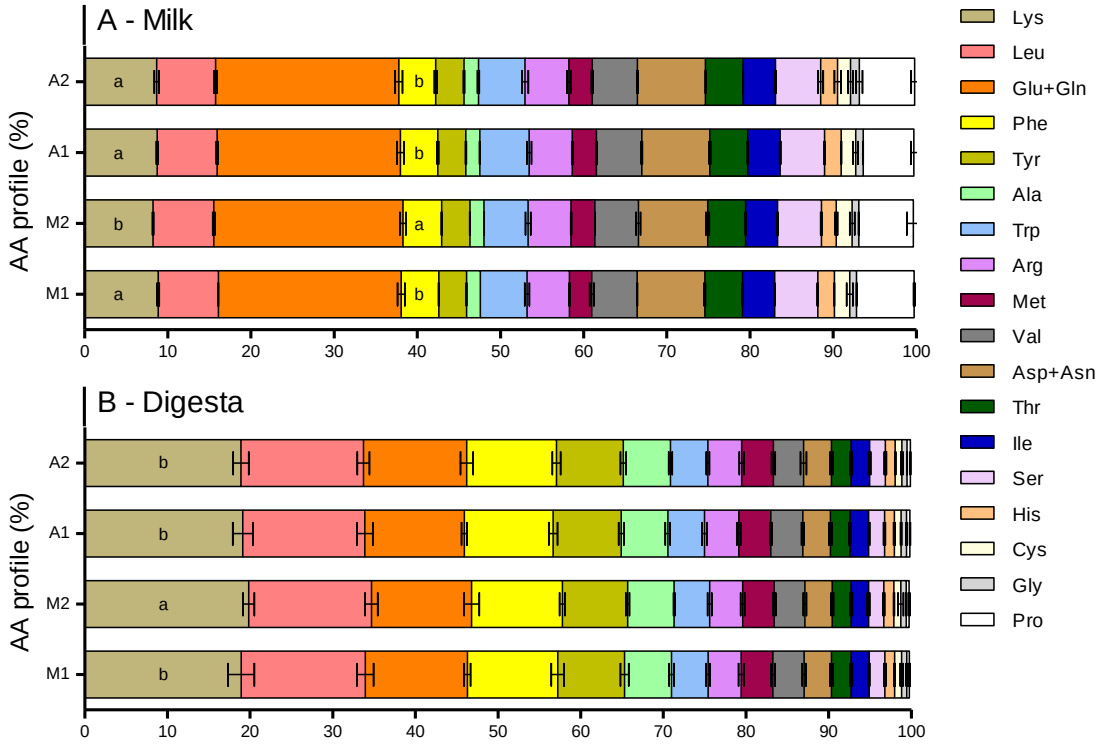

Figure 4.1. Amino acid (AA) profiles: (A) total AA of skimmed UHT milk samples; (B) free AA profile of *in vitro* digested milk samples. Data expressed as mean ± SD of three technical replicates; significant differences are indicated by different letters ($P < 0.05$).

Moreover, there were observed differences for Na ($P < 0.001$), I ($P < 0.001$), Zn ($P < 0.001$), and Cr ($P = 0.01$) contents between the studied milk samples (Table 4.1). Concerning fat content, all samples were below the legal limit of 0.3% (Portaria n. º 473/87, 1987): Mainland 1 and Azores 2 were below 0.1%, Mainland 2 was 0.2%, and Azores 1 was 0.1%.

Table 4.1. Mineral content of the selected milk samples.[a]

	Milk sample				
Mineral group	Mainland 1	Mainland 2	Azores 1	Azores 2	*P*-value
Macrominerals					
Ca	119.4 ± 1.1	120.9 ± 2.5	119.9 ± 3.7	121.2 ± 0.9	NS
Mg	10.5 ± 0.1	10.7 ± 0.3	10.9 ± 0.1	10.9 ± 0.1	NS
K	142.8 ± 1.8	143.6 ± 4.9	136.8 ± 4.2	138.8 ± 1.2	NS
Na	42.3 ± 0.2^{b}	42.3 ± 1.1^{b}	43.7 ± 0.6^{b}	45.8 ± 0.2^{a}	< 0.001
P	98.0 ± 0.9	95.9 ± 4.8	91.5 ± 3.2	92.2 ± 2.0	NS
Microminerals					
Mn	2.51 ± 0.06^{b}	2.18 ± 0.07^{c}	2.94 ± 0.04^{a}	3.01 ± 0.09^{a}	< 0.001
Cu	4.67 ± 0.18^{a}	5.08 ± 0.17^{a}	3.94 ± 0.45^{b}	3.76 ± 0.10^{b}	0.001
Fe	54.9 ± 0.9	53.7 ± 2.2	54.1 ± 2.1	53.8 ± 2.6	NS
I	19.5 ± 0.5^{b}	26.4 ± 0.7^{a}	20.3 ± 0.3^{b}	16.3 ± 0.3^{c}	< 0.001
Zn	400.9 ± 3.1^{a}	391.6 ± 4.5^{ab}	375.5 ± 1.7^{bc}	363.6 ± 6.3^{c}	< 0.001
Se	2.43 ± 0.13	2.77 ± 0.26	2.66 ± 0.25	2.94 ± 0.13	NS
Cr	0.54 ± 0.09^{a}	0.60 ± 0.06^{a}	0.37 ± 0.03^{b}	0.51 ± 0.03^{a}	0.01
Co	0.23 ± 0.01	0.23 ± 0.01	0.22 ± 0.01	0.23 ± 0.02	NS
Mo	2.93 ± 0.06^{a}	3.08 ± 0.11^{a}	2.59 ± 0.06^{b}	2.73 ± 0.08^{b}	<0.001

[a]Macrominerals are expressed in mg 100 g^{-1} milk and microminerals in µg 100 g^{-1} milk. Values represent mean ± SD of three technical replicates; significant differences within a row are indicated by different superscript letters; NS = $P > 0.05$ (*P*-value gives probability of significant differences among treatment groups).

4.3.2. Free AA profile of in vitro *digested milk samples*

Free AA content of 12% TCA-soluble fraction expressed as mg mL^{-1} was similar among digested milk samples (1.82 ± 0.12; 1.84 ± 0.10; 1.83 ± 0.08; 1.80 ± 0.15; $P > 0.05$, respectively for Mainland 1, Mainland 2, Azores 1, and Azores 2). Figure 4.1B shows that all *in vitro* digested milk samples had similar free AA profile, except Mainland 2, which in spite of the lower total content of lysine presented higher relative content of free Lys ($P = 0.01$) after *in vitro* GI digestion.

4.3.3. Effect of H_2O_2 concentration on Caco2/HT-29 co-culture viability

The concentrations 0.2; 0.5; 1.5 and 3.0 mM H_2O_2 were applied to Caco-2/HT 29 co-culture to choose the concentrations to use in subsequent cytotoxicity protection assays as referred

in previous sections. For the concentration of 0.2 mM no effect on cell viability was observed, whereas for the other concentrations reduced cell viability compared with the control was noticed ($P < 0.05$). The concentration of 3.0 mM reduced cell viability to about 50% versus the control ($P < 0.001$; Figure 4.2).

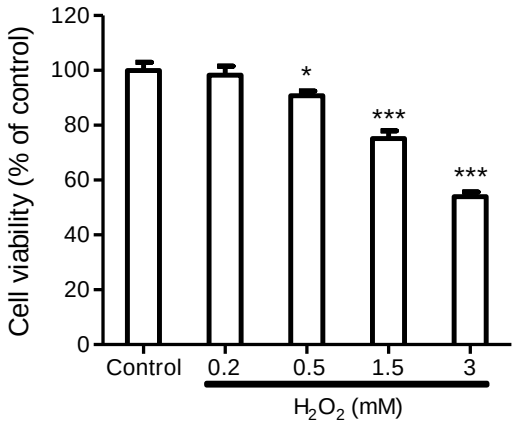

Figure 4.2. Effect of different H_2O_2 concentrations (0.2; 0.5; 1.5; and 3 mM) on Caco2/HT-29 co-culture viability; control corresponds to co-culture not exposed to H_2O_2. Data expressed as mean ± SEM of three independent experiments (*$P < 0.05$, ***$P < 0.001$).

4.3.4. Effect of in vitro *digested milk against induced cytotoxicity*

Figure 4.3A shows that Caco2/HT-29 co-culture viability was not affected by the *in vitro* digested milk. Concerning the effect of milk against induced cytotoxicity, the pre-treatment of Caco2/HT-29 co-culture exposed to 3 mM H_2O_2 with *in vitro* digested milk had no protective effect at the mitochondrial activity level ($P < 0.001$). No significant differences were observed on the cell viability of 3 mM H_2O_2-stimulated cells pre-treated with digested milk and the cells not exposed ($P > 0.05$; Figure 4.3A). Moreover, Figure 4.3B shows that Caco2/HT-29 co-culture exposed to 3 mM H_2O_2 had an increase in caspase-3/7 activity compared with the control (medium only, $P < 0.05$). This increase was similar in cells pre-treated with *in vitro* digested milk and cells not pre-treated, demonstrating that milk fails to attenuate apoptosis via suppression of activated caspase-3/ 7. Figure 4.3B also presents the effect of *in vitro* digested milk on the basal caspase-3/7 activity (not exposed to H_2O_2), evidencing that milk has a neutral effect in an *in vitro* model of the intestinal epithelium under physiological conditions ($P > 0.05$). The enzyme-blank was also applied to co-culture without and with 3 mM H_2O_2 and no effect on cell viability and caspase-3/7 activity was observed (data not shown).

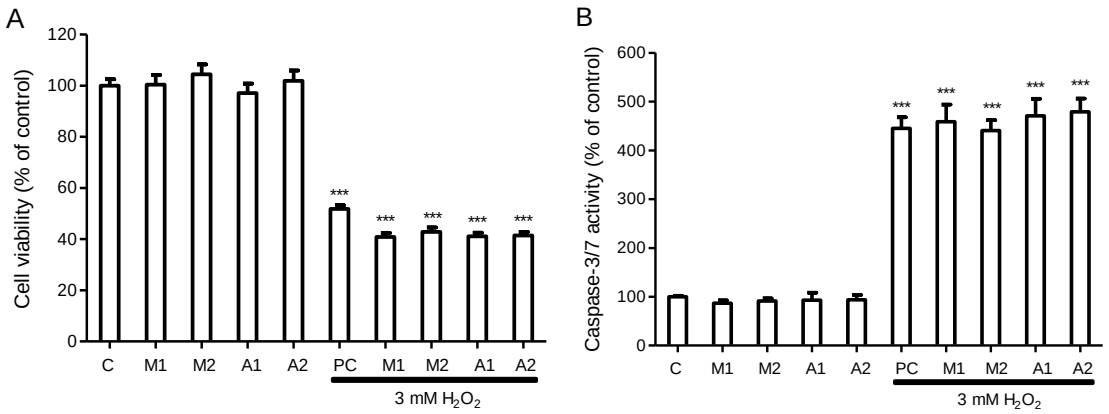

Figure 4.3. Effect of *in vitro* digested milk against induced cytotoxicity of Caco2/HT-29 co-culture by exposure to 3 mM H_2O_2: (A) cell viability by MTT assay; (B) caspase-3/7 activity: C, control (not exposed to H_2O_2, medium only); PC, medium + H_2O_2; M1, Mainland 1; M2, Mainland 2; A1, Azores 1; A2, Azores 2. Data expressed as mean ± SEM of three independent experiments (***P < 0.001).

4.3.5. Effect of in vitro *digested milk on protecting higher ROS level*

The pre-treatment of Caco2/HT-29 co-culture exposed to H_2O_2 with *in vitro* digested milk protected co-culture against increased ROS level because no significant differences were observed when comparing the percentage of ROS formed on 0.2 mM H_2O_2-stimulated cells pre-treated with digested milk and on the control (Figure 4.4), considering that cells stimulated with 0.2 mM H_2O_2 (PC), without the protective effect of milk, showed increased ROS level statistically different from the control (C). Figure 4.4 also presents the effect of *in vitro* digested milk on the basal level of ROS (not H_2O_2- stimulated), showing that milk has a neutral effect in an *in vitro* model of the intestinal epithelium under physiological conditions however protecting it from oxidative stress. The effect of the enzyme-blank on the cellular level of ROS without and with 0.2 mM H_2O_2 was similar to the control (data not shown).

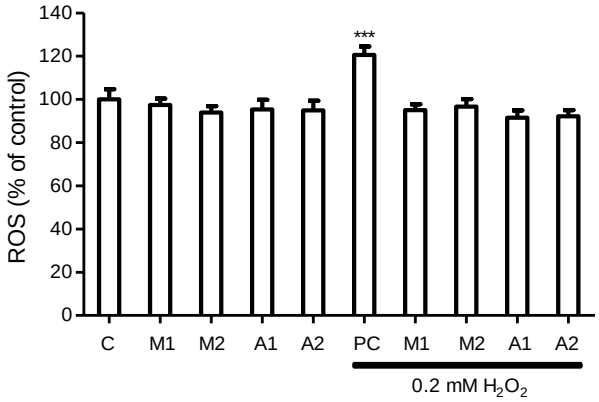

Figure 4.4. Reactive oxygen species (ROS) levels on intestinal Caco2/HT-29 co-culture without (control) and with *in vitro* digested milk and the effect of milk on ROS levels in H_2O_2-stimulated cells

(0.2 mM): C, control (not exposed to H_2O_2, medium only); PC, medium + H_2O_2; M1, Mainland 1; M2, Mainland 2; A1, Azores 1; A2, Azores 2. Data expressed as mean ± SEM of three independent experiments (***P < 0.001).

4.3.6. Effect of in vitro *digested milk on induced NO production*

The pre-treatment of LPS-stimulated RAW264.7 cells with *in vitro* digested milk reduced the induced NO production (P < 0.001; Fig. 4.5). Moreover, cells treated with either medium or enzyme-bank or *in vitro* digested milk only (not LPS-stimulated) presented undetectable levels of NO production and the viability of RAW264.7 macrophage cells was not affected by a composite sample of *in vitro* digested milk neither LPS at 1 mg mL^{-1} (data not shown).

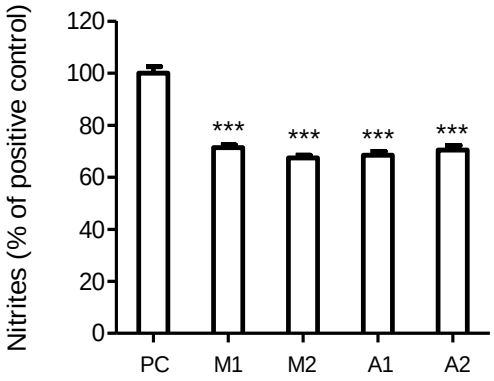

Figure 4.5. Effect of *in vitro* digested milk on nitrite production by LPS-induced RAW264.7 cells: PC, positive control; M1, Mainland 1; M2, Mainland 2; A1, Azores 1; A2, Azores 2. Data expressed as mean ± SEM of three independent experiments (***P < 0.001).

4.4. Discussion

The biological activities of milk-derived compounds have been intensively described in the literature; however, there are still gaps in the current knowledge about bioactivities of the dairy products, particularly in the effect of fluid milk on GI tract (Cilla et al., 2013; Giromini et al., 2019; Thorning et al., 2017).

The present work studied the effect of skimmed UHT milk after simulated human GI digestion in protecting the intestinal tract via preventing induced cell death and higher ROS level in a cellular model of the human intestinal epithelium, as well as attenuating NO production by stimulated macrophages. Although milk contains both hydrophilic and lipophilic bioactive nutrients (Zulueta et al., 2009), in the present study it was used only skimmed milk because the limitations of the harmonised static *in vitro* digestion protocol described by Minekus et al. (2014) regarding fat digestion. This protocol uses constant pH = 3 in the gastric phase favouring pepsin activity. However, the optimum pH of gastric lipase

is between pH 4 and 6 and gastric lipolysis accounts up to one third of fatty acids release (Brodkorb et al., 2019).

Despite *in vivo* models being considered more reliable, a combined model of human simulated digestion with cell lines provides a good representation of *in vivo* conditions and involves fewer ethical restrictions (Egger et al., 2019; Giromini et al., 2019). The intestinal 70/30 Caco2/HT-29 co-culture model proposed by Ferraretto et al. (2018) was selected, because it is considered more suitable than Caco-2 model due to the presence of more than one prevalent intestinal cell type (Ferraretto et al., 2018; Vieira et al., 2016). To study the effect of digested milk in preventing induced NO formation, the model LPS-stimulated RAW264.7 macrophage cells was chosen because these cells are widely used to study the potential interaction of bioactive dietary food components with induced NO production, since an iNOS is expressed in RAW264.7 cells (Chung, Kim, Kim, & Yoo, 2017; Mu et al., 2019). This study was carried out with milk samples from four commercial brands, which represent the main milk players in Portuguese market; moreover, they represent two dairy regions with different geographical features. Thus, the differences observed on AA profile and minerals of those skimmed milks are relevant to confirm that the main outcomes of this study apply to a broad range of skimmed milk samples. The differences in the milk microminerals composition between dairy regions may be related to the chemical composition of the soils where forages are grown (Bhanderi, Goswami, Garg, & Samanta, 2016). Particularly, the Azores Islands are of volcanic origin and the pedogenesis of volcanic rocks can determine Cu (Amaral, Cruz, Cunha, & Rodrigues, 2007) and Mn (Linhares et al., 2019) availability. Minerals content of milk can influence its antioxidant capacity, because some minerals have peroxyl radical-scavenging capacity, namely Mn (Coassin, Ursini, & Bindoli, 1992); others are either an antioxidant or a prooxidant in cellular systems, such as Zn (Lee, 2018), Cr and Mo (Terpilowska & Siwicki, 2019), while Cu is considered an oxidising agent (Bhattacharyya et al., 2014).

The higher relative content of Phe and lower relative content of Lys of milk sample Mainland 2 may be due to genetic factors (Caroli, Chessa, & Erhardt, 2009) or chemical modifications during processing (Mehta & Deeth, 2016). At the end of *in vitro* digestion, the free AA content measured on 12% TCA-soluble fraction was around 57% of the total AA. This amount is in the range described in previous results from *in vitro* digestion (Egger et al., 2019). The free AA profile of all samples was quite similar, only free Lys presented higher relative content (P = 0.01) on Mainland 2. Lys, Phe, Tyr and Trp were abundant in the digested fraction of skimmed milk. Phe and Lys present high superoxide radical scavenging activity, but an intermediate antioxidant activity when considering various antioxidant capacity assays (Guidea, Zagrean-Tuza, Moț, & Sârbu, 2020). Moreover, according to

Tagliazucchi et al. (2016) Trp and Tyr seemed fundamental in the $ABTS^{+}$ and hydroxyl radical scavenging capacities of several identified bioactives peptides and free AA from skimmed milk after *in vitro* GI digestion.

Physiologically, milk compounds released during digestion are found in the intestinal tract after milk consumption and interaction of its compounds occur, independently of some natural compositional variation; therefore, cell assays were performed using *in vitro* digested milk without fractionation. Modulating ROS formation by dietary factors is crucial to avoid cytotoxic ROS levels in cells, namely free AA and peptides (released by GI digestion of proteins) can play an active role in redox reactions. Under the conditions of this study, the increase of basal level of ROS induced by a non-cytotoxic dose of H_2O_2 was completely prevented by the treatment of Caco2/HT-29 co-culture with *in vitro* digested milk before the stimulus, suggesting that after GI digestion skimmed UHT milk can act as an antioxidant. Free AA of the digested fraction, such as Lys, Phe, Tyr and Trp could have contributed to neutralise ROS formation. This finding is in agreement with other studies that evaluated the antioxidant activity of milk after *in vitro* GI digestion using chemical-based methods (Tagliazucchi et al., 2016) and identified Tyr, Trp, and some tyrosine-containing peptides, as responsible for the radical scavenging activity of digested milk based on the Trolox assay, using $ABTS^{+}$ as an oxidant. Additionally, a study with other dairy product (cheese) subjected to *in vitro* digestion, also showed that dairy products have good values of total antioxidant capacity (Barac et al., 2019).

Moreover, 3 mM H_2O_2 was used as an apoptosis inducer of Caco2/HT-29 cells. H_2O_2 triggers apoptosis through the mitochondrial pathway, which requires a cascade of events beginning with altered mitochondrial function and resulting in activation of caspases; caspase-3/7 regulate the execution phase of apoptosis (Xiang, Wan, Guo, & Guo, 2016). Skimmed milk after *in vitro* digestion was not able to attenuate H_2O_2-induced cytotoxicity, as measured by the MTT (which determines the mitochondrial oxidation-reduction activities) and the caspase-3/7 activity assays. These results are in agreement with García-Nebot et al. (2011) and Laparra et al. (2008), who reported that casein phosphopeptides obtained by simulated GI digestion fail in protecting intestinal cells against severe (i.e., 50% reduced cell viability using MTT assay) mitochondrial damage induced by H_2O_2.

Nitric oxide production by iNOS is an inflammatory response to damaged cells. However, sustained overproduction of NO as a result of iNOS upregulation may cause more injury to cells through the formation of highly cytotoxic NO-reactive products (Chokshi et al., 2008; Mu et al., 2019). Bamdad et al. (2017) reported that the peptides released from the whey protein β-LG by high hydrostatic pressure-assisted enzymatic hydrolysis can lower NO production by LPS-stimulated RAW264.7 macrophages cells. In a similar way, the pre-

treatment of LPS-stimulated RAW264.7 cells with *in vitro* digested skimmed UHT milk reduced NO production ($P < 0.05$). Moreover, some bioactive compounds present in milk are also able to reduce LPS-induced inflammatory responses. For example, Calvello et al. (2016) investigated the effect of cell incubation with milk without *in vitro* digestion on NO release and observed reduced LPS-induced NO production. Specifically, these authors were interested in milk isoflavones, which are derived from forage legumes ingested by cows.

4.5. Conclusions

The present study highlights the potential of milk consumption in the protection of intestinal tract from triggers that stimulate ROS and NO formation, since it was observed the neutralisation of increased ROS level in intestinal epithelial cells upon exposure to low-dose H_2O_2, as well as attenuation of NO formation in LPS-activated macrophages, by skimmed UHT milk after *in vitro* digestion. Minerals, free AA and peptides can play an active role in this protective effect, notwithstanding the slight composition difference did not affect the matrix bioactivity after digestion. However, *in vitro* digested milk had no protective effect against induced cell death when intestinal epithelial cells were exposed to high cytotoxic doses of H_2O_2.

Credit author statement

Susana C. M. Pinho: Conceptualization, Formal analysis, Investigation, Writing – Original Draft. **Miguel A. Faria:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – Review & Editing. **Armindo Melo:** Methodology, Investigation, Writing – Review & Editing. **Edgar Pinto:** Methodology, Investigation, Writing – Review & Editing. **Agostinho Almeida:** Resources, Writing – Review & Editing. **Rui Alves:** Project administration. **Ana R. J. Cabrita:** Writing – Review & Editing. **António J. M. Fonseca:** Writing – Review & Editing, Project administration. **Isabel M. P. L. V. O. Ferreira:** Conceptualization, Resources, Supervision, Writing – Review & Editing, Project administration, Funding acquisition.

Declaration of competing interest

None.

4.6. Acknowledgments

Susana C. M. Pinho thanks Fundaçao para a Ciência e a Tecnologia, SANFEED Doctoral Programme, and SORGAL (Sociedade de Óleos e Rações S.A., S. João Ovar, Portugal) the Ph.D. grant PD/BDE/135539/2018. Miguel A. Faria acknowledges FCT/MEC - Fundação para a Ciência e Tecnologia, Ministério da Educação e Ciência - the researcher contract.

This work was supported by FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operational Programme for Competitiveness and Internationalisation (POCI), and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia in the framework of the project PTDC/SAU-NUT/30322/2017.

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PART III

CHAPTER 5 – Overall discussion

Milk nutrients can have a positive or negative impact on health promotion and disease prevention, but there are still gaps in the current knowledge concerning the bioactivity of the dairy products as a complete matrix, particularly about the effect of drinking milk from different geographic origins and feeding production systems in gastrointestinal health. The work herein presented started with the study about the susceptibility of homogenized UHT whole conventional and pasture-based milks to gastric lipolysis and lipid oxidation (**Chapter 2**). Then, it was evaluated the impact of different milk samples in cellular models of gastrointestinal epithelium after *in vitro* digestion. Focus was placed on the capacity of UHT whole (conventional and pasture-based) *versus* fat-free milk to induce gastric epithelial inflammation (**Chapter 3**) and on the potential of UHT skimmed milk from mainland Portugal and the Azores to prevent intestinal cellular redox imbalance by neutralizing high level of reactive oxygen species and attenuate nitric oxide production, as well as the potential protective role of minerals, free amino acids, and peptides in this process (**Chapter 4**).

5.1. Gastric lipolysis and lipid oxidation of whole conventional and pasture-based milks and impact on gastric epithelium

The use of the semi-dynamic INFOGEST protocol to follow the gastric lipolysis and lipid oxidation of UHT whole milk was of great relevance from a methodological perspective because it was demonstrated, for the first time, its applicability to detect differences in the gastric behaviour of conventional and pasture-based milks. Specifically, differences in the relative release of individual fatty acids support that its specific positions on milk triacylglycerols backbone influences the differential digestibility of milk fat from conventional *versus* pasture-based farming.

Moreover, it was observed that the amount of C8:0, C18:2n-6, C18:3n-3, and CLA-*cis*9*trans*11 released during semi-dynamic *in vitro* gastric digestion was higher for the pasture-based milk than the conventional milk, in spite of the similar proportion of C8:0 and lower proportion of C18:2n-6 in the milk composition analysis. This different pattern of free fatty acids reaching the duodenum influences satiety responses once fatty acids up to C12:0 are absorbed directly into the portal circulation, being readily oxidized in the liver (St-Onge & Jones, 2002), and fatty acids with a chain length of >12C are effective releasers of cholecystokinin by the enteroendocrine cells (Rehfeld, 2021).

Satiety plays an important role in the control of further digestion, since it is associated with delayed gastric emptying rate (Goyal, Guo, & Mashimo, 2019). Nevertheless, the influence of satiety degree on milk digestion kinetics was not studied, because the semi-dynamic INFOGEST protocol recommends that gastric emptying dynamics be calculated based on

the delivery of 2 kcal/min; moreover, the initial and final conditions of the simulated stomach should mimic the fasted state (Mulet-Cabero et al., 2020).

The stomach initiates milk fat digestion and fatty acids are in contact with gastric epithelium before moving into the duodenum. In metabolically active tissues, such as the intestinal epithelium, fatty acids exhibit signalling functions, regulating pathways essentially intertwining metabolism and inflammation through free fatty acids receptor-mediated signalling (Kimura, Ichimura, Ohue-Kitano, & Igarashi, 2020; Miyamoto et al., 2016). Nonetheless, information concerning the impact of milk on gastric cells function is very limited. Based on this research gap, in the present study, a gastric epithelial model (NCI-N87 monolayer) was treated with UHT fat-free, whole conventional, and whole pasture-based milks (after semi-dynamic *in vitro* digestion) over 5 days (i.e., mimicking long-term intake) and the mRNA expression of genes related to membrane fatty acids receptors (GPR41 and GPR84), antioxidant enzymes (catalase, superoxide dismutase, and glutathione peroxidase), inflammatory transcription factor NF-κB p65, and pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) was assessed.

Literature suggests that high intake of fat contributes to oxidative stress and inflammation (GBD, 2016), which are closely related pathophysiological processes (Biswas, 2016). Accordingly, results showed that whole milks up-regulated the mRNA expression of catalase, NF-κB p65, and IL-1β, while fat-free milk only up-regulates the mRNA expression of catalase. Therefore, by combining the semi-dynamic INFOGEST method with NCI-N87 cells multiple exposure, it was demonstrated *in vitro*, for the first time, that gastric epithelial cells respond to milk nutrients availability. Specifically, whole milk induces *in vitro* gastric epithelial inflammation. This suggests that the traditional nutrient-based evidence of fatty acids as contributors to inflammation can be extrapolated to whole milks at the gastric epithelium, as well as strength dietary guidelines recommending the intake of fat-free or skimmed milk rather than whole milk.

Moreover, no differences were observed between whole conventional and pasture-based milks even though their distinct amount of C8:0, C18:2n-6, C18:3n-3, and CLA-*cis*9*trans*11 released during semi-dynamic *in vitro* digestion. Therefore, the difference of fatty acids composition did not affect the matrix bioactivity, which supports that it is not possible to conclude the existence of health benefits from a change from milk obtained by conventional systems to a milk with higher content of some valuable nutrients (EP, 2016b).

Increased levels of inflammatory mediators can lead to detrimental effects on the function of tissues (Duan et al., 2018). However, Agace et al. (1993) observed that epithelial cell lines have a more limited cytokine response to inflammatory stimulation than human peripheral blood monocytes, which contribute to limit the consequences of exposure to stimuli at the epithelium and help maintain the integrity of other tissues.

While low amounts of cytokines appear to be important in the homeostasis of uninflamed gastrointestinal epithelium, its production becomes greater during disease states, which can lead to functional consequences within the epithelium (Stadnyk, 2002). For instance, IL1β causes an increase in epithelial tight junction permeability in an NF-κB dependent manner (Al-Sadi, Ye, Said, & Ma, 2010), which can lead to the passage of harmful luminal contents into the gastrointestinal mucosa and systemic circulation (Onyiah & Colgan, 2016).

The present study showed, however, that after interferon-γ-stimulation of the NCI-N87 monolayer, no differences were observed between the whole milk samples and the blank of digestion for the mRNA expression of pro-inflammatory molecules NF-κB p65 and IL-1β. In addition, the antioxidant enzyme catalase was up-regulated by whole milk digesta in a similar way in both simulated gastric luminal states – normal and interferon-γ-stimulated. According to Gurgul, Lortz, Tiedge, Jörns, & Lenzen (2004), overexpression of catalase protects cells against reactive oxygen species and against toxicity from pro-inflammatory cytokines. Hence, results suggests that a regular whole milk intake does not enhance subsequent inflammatory processes.

5.2. Protective effect of skimmed milk on intestinal epithelium

Intestinal epithelium constitutes the largest exchange interface between the external environment and the body, and maintenance of the intestinal barrier function is thus essential in systemic health promotion and diseases prevention (Camilleri et al., 2019). The maintenance of normal intestinal functioning involves preventing oxidative stress (Perez, Talens-Visconti, Rius-Perez, Finamor, & Sastre, 2017). The potential of milk in the intestinal epithelium protection was also studied herein by assessing the potential effect of milk on decreasing the intracellular level of reactive oxygen species on Caco2/HT-29 co-culture exposed to low-dose hydrogen peroxide, preventing cellular death on Caco2/HT-29 co-culture exposed to high-dose hydrogen peroxide, and decreasing nitric oxide formation in lipopolysaccharide-activated macrophages.

None of the available INFOGEST protocols reproduces the dynamic environment of the intestine, but the INFOGEST 1.0 protocol is physiologically comparable regarding protein hydrolysis with *in vivo* models (Egger et al., 2017). Since skimmed milk was used for the harmonization of the INFOGEST 1.0 protocol, in this study only skimmed milk samples were subjected to simulated gastrointestinal digestion and applied to the selected cellular models. Sampling included brands of UHT milk from two distinct geographic origins – mainland Portugal and the Azores, which presented slight differences in amino acid and mineral composition.

It was observed that the totality of skimmed milk samples neutralised the increased level of reactive oxygen species on Caco2/HT-29 co-culture exposed to low-dose hydrogen peroxide, which is an important finding because an overproduction of these species with depletion of the antioxidant system causes cell death (Forrester, Kikuchi, Hernandes, Xu, & Griendling, 2018). Indeed, results showed that exposing Caco2/HT-29 cells to high-dose hydrogen peroxide activated apoptosis signalling pathways. Breakdown of intestinal barrier function leads to chemotaxis and activation of monocytes/macrophages that express inducible nitric oxide synthase, producing large amounts of nitric oxide (Chokshi, Hunter, Guner, Grishin, & Ford, 2008). Results showed that the totality of skimmed milk samples had no protection against apoptosis induced by a high-dose hydrogen peroxide, but attenuated nitric oxide production by lipopolysaccharide-stimulated macrophage cells.

In the present study, the concentration of several microminerals differed between milk from mainland Portugal and the Azores. “Marca Açores/Azores” is an identity brand of the Autonomous Region of the Azores that highlights the most distinctive attributes of the Azores – nature, high environmental value, diversity and natural exclusivity. Particularly, the Azores Islands are of volcanic origin and the pedogenesis of volcanic rocks can determine the availability of some minerals, such as copper (Amaral, Cruz, Cunha, & Rodrigues, 2007) and manganese (Linhares et al., 2019), which affect the chemical composition of forages ingested by cows and thus microminerals composition of milk. An approach that only focuses on isolated nutrients might lead to conclude that a milk with increased amount of specific nutrients has a different bioactivity than a conventional milk. However, it was observed that milks from different geographic origins have similar bioactivity in the intestinal tract using *in vitro* digested milk without fractionation in a cellular model of intestinal epithelium.

Dairy health-based messaging rarely focuses on gut health. Key messages are often on the contribution of dairy to adequate calcium intake and its function of building and maintaining bones and teeth. Dairy messages with a focus on gut health are related to yogurt with probiotics due to the gut flora benefits (Comerford et al., 2021). Nevertheless, the present study demonstrated *in vitro*, for the first time, the potential of UHT skimmed milk consumption in the protection of intestinal tract from triggers that stimulate non-cytotoxic levels of reactive oxygen species and nitric oxide formation.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The present thesis was performed with the partnership of Soja de Portugal – a group that operates in the agro-food industry. Innovation is one of the main strategic vectors of this company and the work herein presented contributes to the development of scientific knowledge related to the dairy sector. Milk composition is affected by geographic origin features and feeding production systems independently of specific farm factors, but information about the potential impact of those differences on human digestive process and health outcomes is scarce. Therefore, this thesis contributes to a better understanding of the relationship between milk composition and the gastrointestinal health, which will help guide feed producers and dairy farmers searching for new ways to promote milk in public health.

Nutrients have an impact on health and an approach that only focuses on isolated nutrients might lead to conclude that a milk with increased amount of a specific nutrient has a different bioactivity than a conventional milk. However, by combining models of human simulated digestion with gastrointestinal epithelial cell lines, it was demonstrated that homogenized UHT whole conventional and pasture-based milks had similar capacity to induce gastric epithelial inflammation. Moreover, UHT skimmed milk from mainland Portugal and the Azores had similar preventive effect on intestinal cellular redox imbalance.

In terms of the impact of milk macronutrients on health (specifically, presence and absence of milk fat), this thesis strengths dietary messages that recommend the intake of fat-free or skimmed milk rather than whole milk, as homogenized UHT whole milk induced gastric epithelial inflammation in the tested conditions and UHT fat-free milk did not. However, it is unclear what observed effect is preferable, because low amounts of cytokines appear to play an important role in the homeostasis of uninflamed gastrointestinal epithelium.

Despite the findings of the present thesis, the results must be further confirmed using a higher number of samples and more complex models of simulated digestion and gastrointestinal tract. Regarding to the inclusion of more samples, it will be essential to consider different processing treatments, increase the number of brands, and include other dairy foods. In relation to the use of more complex models of simulated digestion, it would be important to employ dynamic *in vitro* models and simulate different satiety levels to explore the influence of satiety degree on dairy food hydrolysis kinetics. Relatively to the use of more complex and physiomimetic models of gastrointestinal tract, it will be crucial the use of organ-on-a-chip technology (namely, gut-on-a-chip), with the inclusion of microbiota, which has greater physiological relevance than two-dimensional (2D) cell culture.

Overall, studies on the health effects of foods from different production systems in humans is a research area that needs further work. Understanding the way different food nutrients are released in the gastrointestinal tract and the outcome of food digesta in the gastrointestinal epithelium is one avenue of study and provides important insights into their role in systemic health, as gastrointestinal epithelium is a protective barrier against environmental threats. The establishment of a link between foods from different production methods and systemic health needs the implementation of complex studies including high throughput *in vitro* omic approaches complemented with studies in humans, either interventional or observational.

As future work, it would be interesting to study the outcome of milk consumption in the gut microbiome. The study of the effects of bioactive molecules on gut microbiota and their environment is a hot topic in food science. On the other hand, one of the hypothesis that has been proposed to explain how humans have evolved to digest milk as adults is related to microbiome (the “galactose gut microbiome health hypothesis”). Therefore, it is conceivable that exploiting the role of milk on microbiome will provide further insight on why the metabolic effects of the whole foods may be different from those of proteins, fats, and micronutrients separately, when considering chronic diseases.

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