UNIVERSITY OF CAMERINO

Doctoral Thesis

Biochemical characteristics of cappuccinos made with high-quality pasteurized milk and UHT milk at different steam injection conditions.

Life and Health Sciences

One Health

PhD Candidate:

Dott. Giuseppe Santini

Supervisors:

Prof. Silvia Vincenzetti

Dott. Lauro Fioretti

XXXIV CYCLE

2018/2019 - 2021/2022

CONTENTS

1. INTRODUCTION

This Eureka project arose from the collaboration between the University of Camerino (UNICAM) and the *Simonelli Group* company (Belforte del Chienti, Macerata, Italy), a company specialized in the production of coffee machines. From this collaboration derived also the "Research & Innovation Coffee Hub" (RICH), a heterogeneous group of researchers who aim to study the chemical-physical, nutritional, organoleptic properties of the products (mainly coffee and cappuccino) obtained with the use of the coffee machines produced by the company, and also to optimize the coffee extraction procedures. The ultimate goal is to obtain high quality final products dispensed by increasing the performance of the coffee machines.

Espresso coffee has, over the years, reached global popularity and appreciation. Most of the Italian coffee machines are destined abroad, considering that 3 out of 4 machines are sold in other countries. There is a continuous commitment to improve the quality of the product on the Italian market and at the same time to spread abroad a greater culture of espresso coffee consumption. The same is true for the cappuccino, which is the most consumed beverage both in Italy and in other countries, for which a continuous improvement of the product is required especially regarding the visual aspect, the foam stability, and the organoleptic properties. In particular, for the international market, milk-based drinks represent about 80% of total drinks (Milk; Flat white, characterized by less foam; espresso; cappuccino). Milk and Flat white require a different foam than cappuccino with thinner bubbles and a more liquid final. Furthermore, in the international market, another aspect to consider is the “take-away milk-based drinks” which, unlike cappuccino that is normally prepared in a glass cup, are prepared often in paper/plastic cup and at higher temperatures (70-75° C) with consequences on the chemical-physical and organoleptic characteristics. With this in mind, innovations in espresso machines are constantly evolving, especially in the direction of ergonomics, energy efficiency, design, and temperature stability, but it is important also to know the changes occurring in milk during the preparation of cappuccino and/or milk-based drinks.

This research project fits in this context, with the main objective of obtaining a cappuccino preparation of high organoleptic quality and with peculiar chemical-physical and nutritional properties. Usually, a traditional cappuccino is made of 25 ml espresso and 100 ml steam-foamed milk growing to a volume of about 125 ml. To get a high-quality final product, the selection of the raw material is very important, the cow milk used should have 3.2% minimum protein content and 3.5 % minimum fats in order to get the perfect balance of fat and proteins that ensures good taste and creamy foam with a good texture.

Furthermore, it is very important the temperature at which the milk is whipped: should not exceed 70° C otherwise the milk takes on a "cooked" flavor due to the "caramelization" of lactose, and the proteins present in the milk undergo a process of denaturation which leads to the exposure of sulfhydryl groups which can alter the taste of the beverage itself. In a high-quality cappuccino, the foam should be compact and creamy, with a fine texture and shiny surface. The foam of the cappuccino is a colloidal dispersion of a gas in a liquid, the liquid is the milk used for cappuccino, while the gas consists of the air and water vapor used to whip the milk. The air droplets can form a colloidal dispersion in the milk thanks to the presence in it of surfactants that in the milk is represented by proteins (mainly caseins) and phospholipids. The stability of the foam depends on the relative concentration of surfactants. The higher it is, the more stable the foam, as the gas droplets are small and well separated from each other. Therefore, if the concentration of fats like triglycerides (which are not surfactants) is low, the foam of the cappuccino is more persistent. In whole milk the percentage of fat is around 3.6% so to obtain a creamy and above all stable foam it is important to be able to disperse as much gas as possible during the heating time due to the steam blown into the milk. This is obtained starting from a milk temperature of about 5°C (refrigerator temperature), as the temperature rises (to reach the temperature of 60°C), the milk proteins are partially denatured, contributing to the stabilization of the bubbles in the cappuccino foam.
Finally, in a high-quality cappuccino, the cream should have a thickness of about one centimeter in the cup; this requires a greater injection of air and steam to be incorporated in the milk in the subsequent phase of micronization of the bubbles at the beginning.

1.1 Milk Definition and Characteristics

As discussed before, the choice of the raw material is very important in order to obtain a cappuccino with excellent organoleptic characteristics, and with a creamy and consistent foam. The milk used normally is from a bovine source, even if cappuccinos are recently prepared also using plant-based beverages in order to meet the needs of people affected by cow's milk protein allergy (CMPA) and vegan people. In the case of using cow's milk, the latter must have precise nutritional characteristics to make a cappuccino with enhanced quality.
It is known that milk is the first food for humans and, due to its nutritional characteristics (including those of its derivatives), it is confirmed as one of the fundamental products for human nutrition of all ages. According to health legislation (EC Regulation 1234/2007), "drinking milk" means "*the product obtained from the regular, uninterrupted and complete milking of animals in good health and nutrition*"; the only word "milk" usually refers to cow's milk. In the case of milk obtained from other

mammals, it is necessary to specify the animal species (for example goat milk, buffalo milk, etc.). Commercial milks differ from each other for the fat content or for the preservation procedure used (in the case of heat treatment, the different temperature used). Based on the amount of fat it is possible to find commercially available:

- **Whole milk** with a fat content at least 3.5%;
- **Partially skimmed milk** with a fat content from 1.5% to 1.8%;
- **Skimmed milk** with a fat content less than 0.5%.

If we consider the preservation procedure used, it is possible to find commercially available:

- **Short-life milk (Pasteurized milk),** where milk has been subjected to a pasteurization treatment at temperatures between 72 °C and 78 °C for times between 15 and 20 seconds (with consequent elimination of pathogenic germs and a reduction in the overall bacterial flora). The shelf life of this type of milk is a few days from the date of packaging.
- **Medium-life milk,** where milk has been subjected to heat treatments at temperatures above 80 °C. The shelf life is 90 days from the packaging date.
- **UHT (Ultra High Temperature) long-life** or "sterilized long-life", where milk has been subjected to heat treatments at temperatures of 116 °C - 120 °C for 20 minutes; the shelf life is 180 days from the date of packaging.

There are also special kinds of milk specifically aimed at certain types of consumers such as lactose-free milk deprived of lactose (milk sugar sometimes not digestible in people with a deficiency or absence of β-galactosidase (or lactase), an enzyme that has the task of digesting lactose, or milk enriched with proteins, vitamins, fatty acids, fibers, and other compounds.

1.2 Milk Nutritional Composition

Bovine milk is composed, on average, of 87% water, 4-5% lactose, 3% protein, 3-4% fat, 0.8% minerals, and 0.1% vitamins, as shown in more detail in **Table 1.**

Table 1. Average nutritional composition of whole, low-fat and skim milk (Adapted from: Pereira, 2014).

Composition (100g)	Whole	Low-fat	Skim (UHT)
Energy (kcal)	62.0	47.0	34.0
Water (g)	88.1	89.1	90.5
Protein (g)	3.0	3.4	3.3
Fat (g)	3.5	1.6	0.2
Carbohydrates (g)	4.7	4.9	4.9
Cholesterol (mg)	13.0	8.0	1.0
Vitamin A (mg)	59.0	22.0	0
Vitamin D (mg)	0.05	0.05	0
Vitamin B1 (mg)	0.040	0.04	0.05
Vitamin B2 (mg)	0.14	0.11	0.05
Na (mg)	43.0	41.0	41.0
Ca (mg)	109.0	112.0	114.0
Mg (mg)	9.0	9.0	10.0

1.3 Milk Protein Fraction

Milk is generally considered an important protein source in the human diet, with 32 g protein/L supplied, on average. The protein fraction is divided into insoluble and soluble proteins. The soluble fraction is represented by whey proteins for 20% of total milk proteins, instead, the insoluble, named caseins, represent 80%. Both are classified as high-quality proteins considering the human amino acid requirements, the bioavailability, and the digestibility. Milk proteins are considered in fact, as the best protein source for human nutrition. The amino acid profile between the two fractions is quite different: whey proteins are especially rich in branched-chain amino acids, isoleucine, valine, and leucine as well as lysine, instead casein has a higher proportion of phenylalanine, methionine, and histidine. Milk proteins and several bioactive peptides derived from their enzymatic hydrolysis have also shown multiple biological roles with protective action in human health. These include antiviral, antibacterial, antioxidant, antifungal, antimicrobial, antihypertensive, opioid, antithrombotic, and immunomodulatory activities, in addition to the role of enhancing the absorption of other nutrients (**Table 2**).

Table 2. Concentration and functional role of the main milk proteins. (Adapted from: Pereira, 2014)

PROTEIN	COW (g/L)	FUNCTION
Total caseins	26.0	Mineral transport (Ca, PO_4, Fe, Zn, Cu)
α-casein	13.0	
β-casein	9.3	
κ-casein	3.3	
Total whey proteins	6.3	
β-lactoglobulin	3.2	Retinol and fatty acid binding, possible antioxidant
α-lactalbumin	1.2	Lactose production, Ca transport, immunomodulator, anticancer agent
Immunoglobulin	0.7	Immune protection
Serum albumin	0.40	
Lactoferrin	0.1	Antimicrobic agent, antioxidant, immunomodulator, iron absorption
Lactoperoxidase	0.03	Antimicrobic agent
Lysozyme	Trace	Antimicrobic agent, synergy action with immunoglobulins and lactoferrin
Others	0.8	
Proteose-peptone	1.2	
Glycomacropeptides	1.2	Antiviral

Whey Protein Functional Role

The soluble protein fraction (whey proteins) includes the following proteins: α-lactalbumin, β-lactoglobulin, bovine serum albumin, lactoperoxidase, lactoferrin, lysozyme, transferrin, and proteose-peptone. Below is a brief description on the structure and function of the main whey proteins in milk which could be helpful later for the discussion of the results obtained in this study.

α-lactalbumin (α-LA)

α-LA (**Figure 1)** is a small protein (Mr 14.2 kDa), with a Ca^{2+} ion binding site. It has been hypothesized that the α-LA gene originated 300-400 million years ago from an ancestral lysozyme gene, per gene duplication. This is the reason why α-lactalbumin shares a 40% sequence identity with lysozyme (also their three-dimensional structure looks very similar). This protein is made up of two domains: a large α-helical domain and another smaller one consisting of a β-sheet secondary structure. These two domains are linked together by a disulfide bridge between the cysteine residues 73 and 91, which also forms the calcium-binding site and another disulfide bridge between 61 and 77 cysteine residues. Definitely, the overall structure of α-LA is stabilized by four disulfide bridges (Permyakova et al., 2000).

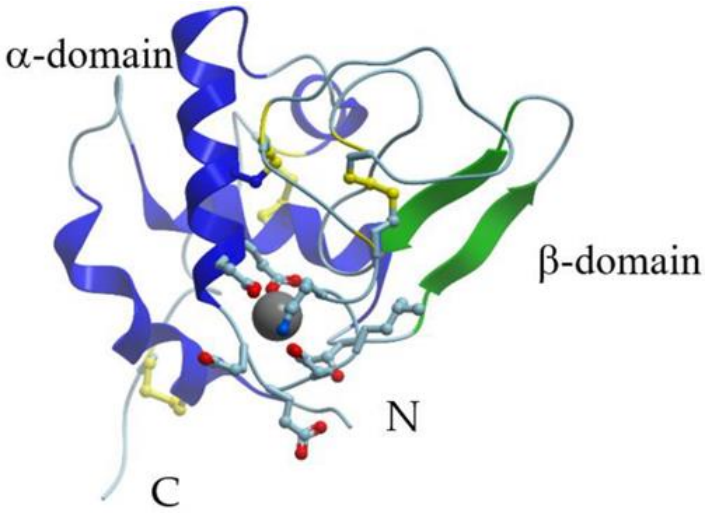


Figure 1: α-LA three-dimensional structure (From: Permyakov, 2020)

This protein is important for various reasons. Firstly, α-LA is part of the enzyme complex that regulates the synthesis of lactose in the mammary gland and also facilitates its absorption of lactose in the intestine. Secondly, α-LA has a host of other functions, such as inflammatory and antinociceptive functions. Some authors have shown that the proteolytic digest of α-LA released three peptides with bactericidal properties. In particular, two fragments were obtained from tryptic digestion and the third was obtained by fragmentation of α-LA by chymotrypsin. These polypeptides were mainly active against Gram-positive bacteria, while they were less active on Gram-negative bacteria. However, if α-LA was digested by pepsin, the resulting polypeptide fragments did not show any antibacterial activity. Since undigested α-LA does not possess bactericidal activity, it has been suggested as a possible antimicrobial function of α-lactalbumin only after its partial digestion by endopeptidases (Pelligrini, et al., 1999). Therefore, these bioactive peptides are buried within the a-LA sequence and are released by the action of proteolytic enzymes. Other authors purified α-LA folding variants from human milk with bactericidal activity against antibiotic-resistant strains of *Streptococcus pneumoniae*. In these experiments, the native α-LA was converted to the active bactericidal form by an anion-exchange chromatography performed in the presence of a C18:1 fatty acid (oleic acid) (Hakansson, et al., 2000). It was in fact demonstrated that native α-LA possesses several classes of fatty acid binding sites (Cawthern, et al., 1997). The resulting complex showed an antibacterial activity but only for Streptococci, in fact, Gram-negative and other Gram-positive bacteria were resistant to this activity. Finally, some multimeric human α-La derivative induces an increase in Ca^{2+} level and acts as an apoptosis-inducing agent (Hakansson, et al., 1995; Svensson, et al., 1999). In fact, it has been shown that multimeric α-LA can bind to the cell surface, enter the

cytoplasm and accumulate in the nuclei. Further, the direct interaction of α-LA with mitochondria leads to cytochrome c release, which, in turn, starts the caspase cascade which is involved in apoptosis (Köhler, et al., 1999). It is known that in human milk the α-lactalbumin-oleic acid complex, called HAMLET (Human α-lactalbumin Made Lethal to Tumor Cells), can induce selective apoptosis towards cancer cells, but leaves fully differentiated cells unaffected. The *in vivo* effects of HAMLET have been investigated in patients and tumor cell lines, and the results indicated that HAMLET limits the progression of human glioblastomas in a xenograft model and removes skin papillomas in patients. In tumor cells, HAMLET enters the cytoplasm and then enters the nuclei where it accumulates. Here, HAMLET binds to histones and disrupts chromatin organization (Mossberg, et al., 2010). Furthermore, it has been shown that the acid pH in the stomach of the breastfed child can promote the formation of HAMLET, which contributes to the protective effect of breastfeeding against childhood tumors (Mossberg, A.K. et al., 2010).

β-lactoglobulin (β-LG)

β-LG (**Figure 2)** is a small protein with 162 amino acid residues (Mr ~ 18.4 kDa) - its sequence and three-dimensionality structure (it is a dimer) show that it is part of the lipocalin family which includes a large and different group of over 50 extracellular proteins that come from a wide variety of tissues in the animals, plants, and bacteria. (Vincenzetti et al, 2017) A typical lipocalin consists of a peptide chain of 160-180 amino acids, folded into 8 antiparallel filaments which produce a β-sheet arranged in a conical structure called β-barrel, in which the hydrophobic pocket is located, capable of binding several hydrophobic molecules.

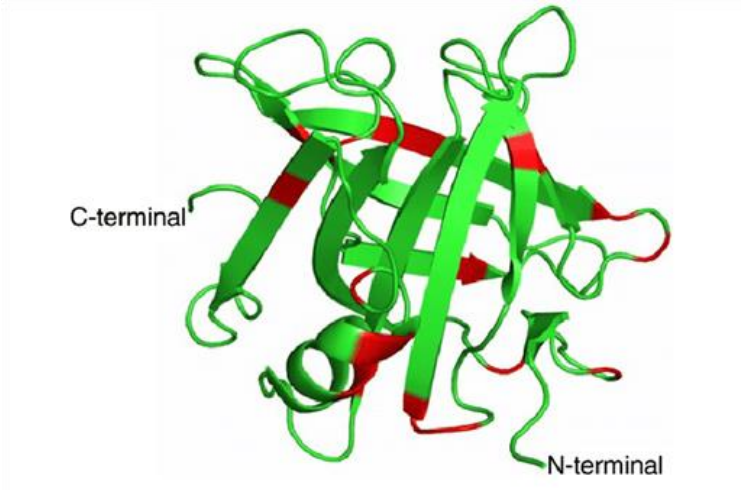


Figure 2: β-LG molecular structure. (From: Cheison et al., 2011)

Indeed, the members of this family are made up of some common molecular properties: the ability to bind several small hydrophobic molecules, the ability to bind to specific cell surface receptors e the ability to form complexes with soluble macromolecules. For these reasons, lipocalins can act as specific transporters, for example, for serum retinol-binding protein (RBP) (Kontopidis et al, 2004). Hydrophobic ligands are mainly represented by long-chain fatty acids, retinol, and steroids. In milk, fatty acids, rather than retinol, represent endogenous ligands; the last one, however, being more soluble in fat, is indirectly transferred from the mother to the infant by the β-LG. Apart from this evidence, the effective role of this protein is not completely known. This is due to two main reasons: the first is given by the presence of other lipocalins that have a similar function, and the second is simple reasoning based on the fact that if β-LG had played an indispensable role it would be expressed in all mammals and not just in some. A hypothesis on the role of this protein could be that the real β-LG function may be related to the physiology of the mother rather than that of the newborn. An important issue was the identification of a pseudogene (ancestral genes that have lost the ability to express themselves), which looked very similar to the equine β-LG-II sequence (Kontopidis et al, 2004). One explanation for the presence of the pseudogene could be that the protein served for its true function elsewhere and can therefore be expressed in the mammary gland of some species (but not all) for nutritional purposes. Examining the β-LG sequences of the various species together with the other lipocalins, a family tree has been realized from which can be noticed that the RBPs are clearly distinct from lactoglobulins, with the exception of a single protein: glycodelin. This is a protein expressed in large quantities during human pregnancy (first three months) in the endometrium and appears to be involved in immunosuppression and/or cell differentiation (Kontopidis et al., 2004). Therefore, it might be possible to postulate that in many species this gene has undergone a duplication event and is now expressed during lactation for nutritional purposes. Likewise, in some species, such as rodents, lagomorphs, and humans, the formation of a pseudogene has ceased, and consequently, it ceased its presence in the milk of these species (Kontopidis et al., 2004). Recently it has been shown that β-LG is able to interact with resveratrol, a natural polyphenolic compound with antioxidant activity that forms a 1:1 complex that enhances the photostability and water solubility of resveratrol, improving its bioavailability (Liang, L. et al, 2010). Also, it has been shown that β-LG is able to form complexes also with folic acid; therefore, it can be used as an effective carrier of this important vitamin in food (Permyakova, E.A. et al, 2000).

Lactoferrin

Lactoferrin (**Figure 3**), also called lactotransferrin, is an 80.0 kDa glycoprotein belonging to the transferrin family, is an iron-chelating glycoprotein and has a structure composed of two homologous domains, each binds a ferric ion (Fe^{3+}) and a carbonate anion.

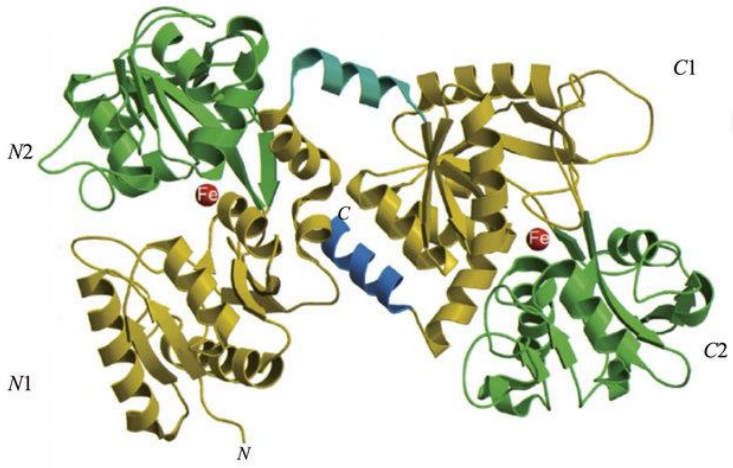


Figure 3: Lactoferrin molecular structure. The ferric ion is represented by red spheres. (From: Rosa et al., 2017)

It is a protein that exhibits several functions, regulation of iron homeostasis, cell growth and differentiation, defense against infections agents, anti-inflammatory and anticancer agents and, finally, a trophic activity in the intestine mucosa (Ward, P.P. et al, 2005). Lactoferrin is mainly present in milk, and also in small quantities in exocrine liquids such as come saliva, tears, bile, seminal fluid, and pancreatic juice. Plasma contains a low concentration of lactoferrin, but during inflammatory reactions, neutrophil granulocytes release this protein increasing the concentration of the plasma pool. The concentration of lactoferrin is quite high in human milk (1.0 mg/ml) compared to bovine (0.02–0.2 g/l), sheep (0.14 g/ml) and goat (0.02–0.40 g/l) milk. However, in all species, the highest concentration of lactoferrin is found in colostrum (in human colostrum is about 7.0 g/l) and may increase in case of breast infection (Vincenzetti et al., 2017). Lactoferrin exhibits two different mechanisms of antimicrobial activity. The first mechanism is due to its high affinity with iron, therefore it can remove this metal from iron-dependent bacteria, which are thus deprived of an essential element for their growth. Thanks to its bacteriostatic properties, and the ability to bind iron, lactoferrin is capable of retarding the growth of a wide variety of microorganisms, including a wide range of gram-positive and Gram-negative bacteria and some types of yeasts. However, the bacteriostatic effect is often temporary because some Gram-negative bacteria have become unable to

adapt to the restrictive conditions of iron through the synthesis of low molecular weight chelating agents (siderophores) which are able to remove iron from lactoferrin. The second antimicrobial activity is due to a direct-acting mechanism of lactoferrin that is capable of damaging the cell wall of Gram-negative bacteria by binding to lipopolysaccharide A, porins, and other cell wall surface molecules of some microorganisms (Vincenzetti et al., 2017). The role of lactoferrin as an important anti-inflammatory agent is associated with the microbial challenge. It has been shown through animal studies that the administration of lactoferrin protects against gastritis by acting against *Helicobacter pylori* (Dial, E.J. et al., 2002). This protective effect against infections seems to be due to the fact that lactoferrin promotes the inhibition of several pro-inflammatory drugs cytokines such as tumor necrosis factor alpha (TNFa), interleukin-1β (IL-1β), and IL-6. Lactoferrin is upregulated in various inflammatory disorders such as neurodegenerative diseases, arthritis, allergies skin, inflammatory bowel diseases, and lung disorders (Ward, P.P. et al, 2005). Also, lactoferrin, in addition to the many biological roles already known, stimulates the proliferation and differentiation of osteoblasts. (Cornish et al., 2006). From the proteolytic digestion of lactoferrin, some peptides are derived with antimicrobial activity against pathogens, namely LF1-11, and lactoferricin. All of these derive from the N-terminal domain of lactoferrin and are conserved in the lactoferrin of most species. The most important peptide is the lactoferricin which exerts its antibacterial activity against numerous bacteria, viruses, fungal pathogens, and protozoa. Furthermore, this peptide showed other activities, such as tumor inhibition metastasis in mice and induction of apoptosis in THP-1 human monocytic leukemia cells (Sinha, M. et al., 2013). Comparing the antimicrobial activities of lactoferricin from humans, cattle, and murine caprine, bovine lactoferricin was shown to be the most active. The minimum inhibitory concentration (MIC) against some strains of *Escherichia coli* was about 30 µg/ml, while in lactoferricin derived from human lactoferrin, it was approximately 4 times higher. The highest antimicrobial activity of bovine lactoferricin seems to be due to the presence of high quantities of net positive charges and hydrophobic residues (Sinha et al., 2013). Another group of peptides that comes from the digestion of lactoferrin includes lactopherroxins. These are classified as opioid peptides, as they showed similar activity to that of opiates (Jenssen et al., 2007). Other opioid peptides can also be obtained from the digestion of caseins (casomorphins).

Lactoperoxidase (LPO)

LPO (**Figure 4**) is an enzyme secreted predominantly by the mammalian gland, but it can also be found in other glandular secretions. This enzyme is part of the peroxidase family and is a glycoprotein

consisting of 608 amino acids with a molecular mass of 78.0 kDa. Its secondary structure consists of α-helices and two shorts antiparallel β sheets which together form a spheroidal structure with a heme group in the center covalently connected. A calcium ion is also bound to the enzyme, important for maintaining the structural integrity of the protein (Tenovuo et al., 1985).

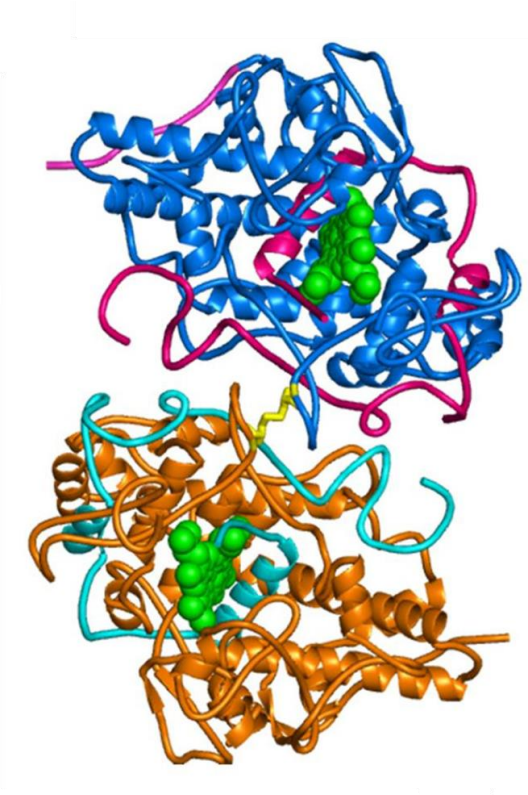


Figure 4: LPO molecular structure (From: Sharma et al., 2013)

The LPO system catalyzes the oxidation of multiple substrates, using hydrogen peroxide (H_2O_2) as shown in the following reaction:

$$\text{Reduced substrate} + H_2O_2 \rightarrow \text{Oxidized product} + H_2O$$

Reduced substrates include thiocyanate (SCN^-) and iodide ions (I^-), while hydrogen peroxide can derive from the reaction of glucose with oxygen catalyzed by the enzyme glucose oxidase. Once formed, the oxidized products have powerful bactericidal activity against bacteria, viruses, parasites, fungi, and mycoplasma (Tanaka et al., 2007). The LPO system also has a bacteriostatic effect on

Listeria monocytogenes, therefore, it should be used to control the development of this bacterium in raw milk refrigeration temperatures. Additionally, some authors co-administered lactoferrin and LPO to mice infected with the influenza virus in order to alleviate pneumonia (Shin et al., 2005). Thanks to its bacteriostatic activity, LPO is widely used in food storage.

Bovine Serum Albumin (BSA)

BSA (**Figure 5**) is not produced in the mammary gland but is secreted in the milk after its passive loss from the bloodstream.

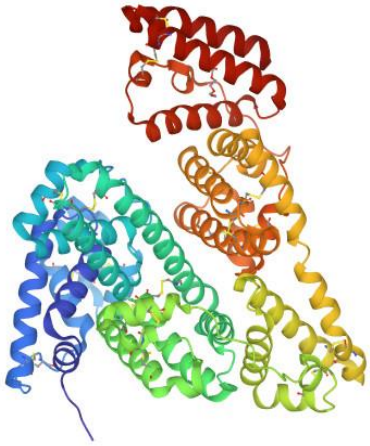


Figure 5: BSA molecular structure. (From: Bujacz 2012)

The most important property of bovine serum albumin is its ability to bind to various ligands reversibly. It is the main carrier of fatty acids and can bind free fatty acids and other lipids, as well as flavoring compounds (Huang et al., 2004). However, this property is lost in case of denaturation induced by heating. BSA also inhibits tumor growth due to the modulation of the activities of autocrine growth-regulating factors and also is involved in the lipid's synthesis (Laursen et al., 1990; Choi et al., 2002). Furthermore, BSA possesses antioxidant activities and is a source of all major essential amino acids (Tong et al., 2000).

Caseins

Caseins are the main protein component of cow's milk, making up about 80% of the total milk proteins. There are four individual types of casein molecules, the caseins αs1-, αs2-, β- and κ respectively in approximate relative quantities of 4:1:3.5:1.5 (Swaisgood, 2003, Farrell et al., 2004). In milk, the caseins, together with the essential ingredient of calcium phosphate (de Kruif & Holt, 2003), form aggregates of several thousand single protein molecules with average diameters from 150 to 200 nm (de Kruif, 1998), known as casein micelles (Fox & Brodkorb, 2008). Micelles are highly hydrated, with about 3.5 kg of water per kg of protein (Jeurnink & de Kruif, 1993). Although the caseins make up about 2.5% of the total weight of milk, micelles take up about 10% of the volume. Micelles can be moderately heated or cooled without significant aggregation or disruption of their basic structures. On the other hand, they are easily destabilized by treatment with proteolytic enzymes or by acidification to give the clots which are in cheese bases and products such as yoghurt. Most of the functional properties of micelles depend on the properties of the surface, rather than the inside, and to some extent micelles can be considered hard spheres with a protective coating (de Kruif, 1999). On the other hand, the inner part of the micelles becomes important in such post-coagulant rearrangements as the formation of cheese curd. Besides, there is the possibility of using micelles as carriers of nutritionally significant molecules, and for this, an understanding of the inner part, highly hydrated, can be important (Dalgleish & Corredig, 2012). Several studies have shown that smaller micelles are relatively rich in κ-casein and relatively depleted of β-casein, while the αs-casein content appears to be independent of size (Dalgleish et al. 1989, Marchin et al. 2007). These results imply that κ-casein is predominantly if not completely on the surface of the micelle, β-casein is mostly present inside and the αs-caseins are found throughout the structure. (Dalgleish & Horne, 1985). The size of native micelles is determined by the amount of surface area that can be stabilized by the available κ-casein; therefore, the greater the proportion of κ-casein present in the total proteins, the smaller the micelles (Delacroix-Buchet et al. 1993). The average size depends on the composition of the milk, but, in general, the average hydrodynamic diameters are from 150 to 200 nm. Size distribution analysis by field-flow fractionation techniques has suggested a minimum diameter of about 80 nm (de Kruif, 1998), although some particles may be up to 50 nm in reconstituted milk from skimmed milk powder (Udabage et al. 2003). From relatively simple coat-core models of the αs- and β-caseins surrounded by a layer of κ-casein (Waugh et al. 1970), structural models have been developed to provide two contenders for this structure (**Figure 6**), namely sub-micellar and nanocluster models (Farrell et al. 2006, Horne, 2006). the sub-micellar model was first hypothesized. It follows from the observation that the protein in sodium caseinate (the protein extract from micelles when calcium phosphate has been removed by acidification) forms small aggregates via non-covalent

interactions when they are dispersed in aqueous media (Waugh et al., 1970). It was therefore a simple hypothesis postulated that the micelles were formed from these aggregates (sub-cells) linked together by small domains of calcium phosphate (Schmidt, 1982). The knowledge that κ-casein is mainly a being on the surface of the micelles implies that at least two types of sub-cells (rich κ-casein and lean κ-casein) must exist. The alternative model, nanocluster, is derived from the observation that phosphopeptides from β-casein are able to stabilize calcium phosphate in solutions at concentrations that would precipitate (Little & Holt, 2004). The study of this phenomenon showed that the phosphopeptides bound and stabilized small domains of calcium phosphate, called nanoclusters. The calcium phosphate nanoclusters have a radius of 2.3 nm and are surrounded by approximatively 50 phosphopeptide chains (Holt et al., 1998).

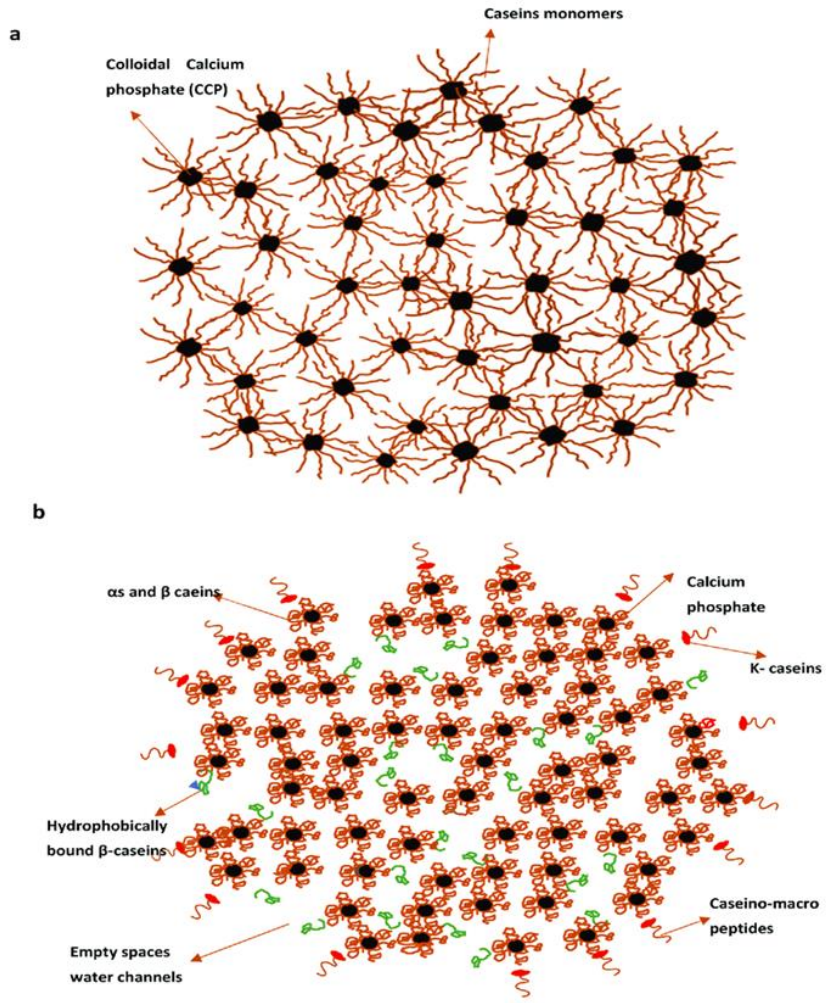


Figure 6: (a) Casein micelle models by Holt; (b) casein micelle models by Dalgleish. (From Sadiq et al., 2021)

From casein hydrolysis by proteolytic enzymes the bioactive peptides are derived that show multifunctional properties. In fact, they initially act as physiological modulators of metabolism during

intestinal digestion of food and, once absorbed, can act on the various target organs of the body. In particular, bioactive peptides play various roles in the cardiovascular, digestive, nervous, and immune systems (Silva et al., 2005). Some authors have shown that some peptides derived from whey and casein proteins possess ACE-inhibitory activity, in particular, the identified peptides come from αs1-casein digestion (fragments 142–147, 157–164 and 194–199) and β-casein digestion (fragments 108–113, 177–183 and 193–198) (Pihlanto-Leppala et al., 1998). *In vitro* enzymatic hydrolysis of β-casein produces several peptides with opioid-like activity and is characterized by a common N-terminal Tyr-Gly-Gly-Phe sequence, where the presence of a Tyr residue at the N-terminal is important for opioid activity. The most important opioid peptides are the β-casomorphins, which are fragments of β-casein between the 60th and 70th residue (Silva et al., 2005). The presence of these bioactive peptides has an effect on the central nervous system (analgesic and sedative action) and on the endocrine system. Furthermore, casein-derived bioactive peptides are also immunomodulatory activity (stimulation of the immune system) and antimicrobial activity (inhibition of pathogens). It has been shown that the digestion of caseins in human and bovine milk releases peptides with immunostimulating activity, in particular the immunomodulatory activity of the fragment 54-50 of human b-casein (Hexapeptide Val-Glu-Pro-Ile-Pro-Tyr). (Parker et al., 1984)

1.4 Milk Protein Denaturation

The whey proteins are typical globular proteins with well-defined secondary and tertiary structures. These proteins, (especially α-LA and β-LG) maintain their native conformations only within relatively limited temperature ranges. The exposure of whey proteins to extremes of temperature results in the denaturation and aggregation of the proteins (Anema, 2020). When the native protein is in the form of non-covalently bound oligomers, such as dimeric β-LG, the first step in the denaturation process is the dissociation of the oligomer into monomeric species (Anema, 2020). In principle, this step is reversible; however, in complex mixtures such as milk, the unfolding process is accompanied by the exposure of reactive amino side chain groups, which allow irreversible aggregation reactions to occur. The unfolded whey proteins can aggregate with each other, or with the casein micelles (Anema, 2020). α-LA is generally considered to be one of the most heat-labile whey proteins, whereas β-LG is one of the most heat-stable whey proteins, however, for the dairy industry, it is the irreversible aggregation processes that largely determine the functional properties of dairy products (Ruegg et al., 1977). In general, significant denaturation of the major whey proteins, α-LA, and β-LG occurs only by heating the milk to a temperature above 70° C (Anema, 2020). One of the main reactions of interest

is the interaction between denatured β-LG with κ-casein (κ-CN) on the surface of the micelles. Some studies indicated that this interaction between β-LG and κ-CN occurs when these components are heated together (Zittle et al., 1962; Long et al., 1963; Sawyer et al., 1963). These conclusions have been drawn from electrophoretic studies, which showed that the bands assigned to the κ-CN and β-LG observed in unheated solutions produced intermediate mobility species when the solutions were mixed and heated together (Zittle et al., 1962). Subsequent investigations in heated model systems, determined the types of the bonds involved in the complexes formation, their stoichiometry, and the involvement of other whey proteins (in particular α-LA) in the complexes. It was shown that the presence of reducing agents dissociated the heat-induced complexes and that the thiol blocking agents prevented the complex formation (Sawyer et al., 1963). These results supported the hypothesis that the free thiol group of β-LG was involved in the interactions, and that intermolecular disulfide bonds were formed between κ-CN and denatured β-LG (Zittle et al., 1962; Sawyer et al., 1963). It is now generally believed that interactions between α-LA and κ-CN will occur if β-LG (or another whey protein with a free thiol group exposed) is present during heating, and this may require the initial formation of a β-LG-α-LA complex, which subsequently interacts with κ-CN (Baer et al., 1976; Elfagm & Wheelock, 1978). A recent study demonstrated the importance of thiol-disulfide interactions in the irreversible denaturation and aggregation of α-LA in milk and whey protein solutions (Nguyen et al., 2018). In this work, it was shown that the irreversible thermal denaturation of α-LA was promoted when a low amount of a thiol reagent (Cysteine hydrochloride or reduced glutathione, or 2-mercaptoethanol) was added to the milk, with significant denaturation occurring at temperatures up to 60°C. The thermal denaturation of β-LG was also enhanced with the addition of these thiol reagents, but at a higher temperature (above 70° C). These authors proposed that, at temperatures between 61 and 70° C, the added thiol reagents begin thiol-disulfide exchange reactions with the disulfide bonds of the unfolded α-LA, thus favoring irreversible aggregation reactions. At temperatures above 70° C, the thiol reagent, together with the free thiol of the unfolded β-LG, started thiol-disulfide interchange reactions with the disulfide bonds of both α-LA and β-LG, thus promoting the irreversible aggregation of these proteins (Nguyen et al., 2013; Nguyen et al., 2018). In the following scheme, is shown the proposed mechanism by Nguyen and co-workers (2018) for α-LA and β-LG denaturation:

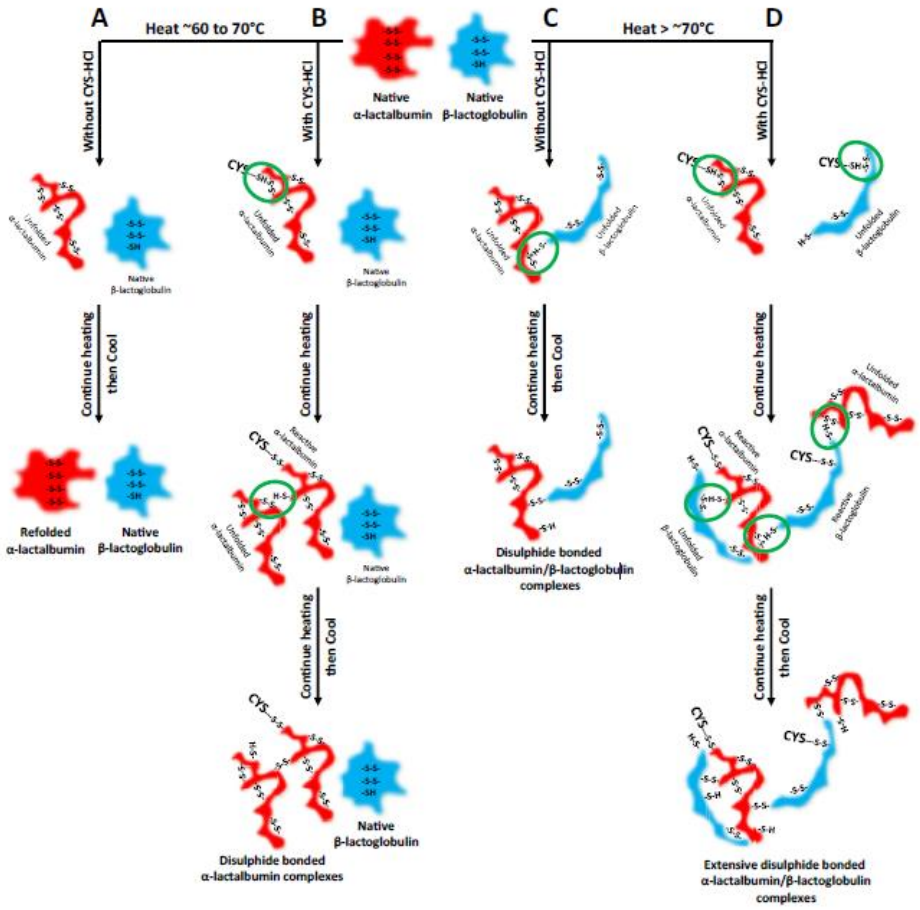


Figure 7. β-LG and α-LA denaturation process. (Nguyen et al., 2018)

When temperatures are above the denaturation temperature of β-lactoglobulin but in absence of the thiol reagent (Cysteine hydrochloride, Cys-HCl), both α-LA and β-LG unfold. b-lactoglobulin expose the proper thiol groups that start the thiol-disulfide exchange reactions between α-LA and β-LG and as a consequence, these two proteins are irreversibly denatured and the subsequent cooling reduce the amount of the native protein (Nguyen et al., 2018)

There are considerable evidence showing that disulfide bonds are involved in the aggregates formed between denatured whey protein and κ-CN; however, there are reports that suggest that other non-covalent bonding may be important in these interactions, particularly in the early stages of heating and at lower heating temperatures (Sawyer, 1969; Haque et al., 1987; Haque & Kinsella, 1988; Collina, 1989). It was suggested also that both hydrophobic and disulfide interactions are important in the early stages of aggregate formation, with the interaction mechanism depending on the composition of the system and heating conditions (Cho et al., 2003). Studies on the interactions between proteins in heated milk suggest that despite the complexity of the system, the reactions between β-LG and κ-CN may be similar to those that occur in model systems (Anema, 2020). The degree of interaction of denatured whey proteins with casein micelles depends on many variables

including time, temperature, and rate of heating; milk and individual protein concentrations; the pH of the milk; and the concentration of milk salts (Smits & van Brouwershaven, 1980; Corredig & Dalgleish, 1996a, b; Oldfield et al., 2000; Anema & Li, 2003b; Oldfield et al., 2005). For example, when the temperature of the milk it is gradually increased above 70°C, as in indirect heating systems, most of the denatured b-LG and α-LA associate with casein micelles, presumably as disulfide-bound complexes with κ-CN on the surface of the micelles (Smits & van Brouwershaven, 1980; Corredig & Dalgleish, 1996b). Conversely, when the milk is heated rapidly, such as in direct heating systems, only about half of denatured β-LG and α-LG is associated with casein micelles (Singh and Creamer, 1991a; Corredig & Dalgleish, 1996a; Oldfield et al., 1998b).

1.5 Fatty Acid Fraction

Fat fraction inside milk is represented by the milk fat globules (MFG) that are resistant to lipolysis by pancreatic enzymes unless they are first submitted to gastric digestion. 98% of milk fat fraction is represented by triacylglycerol (TAG), the other lipids are represented by diacylglycerol (2%), phospholipids (1%), cholesterol (< 0.5%), and free fatty acids (0.1%). Additionally, there are also traces of hydrocarbons, flavor compounds, fat-soluble vitamins, and other elements introduced through the animal feed. Milk fat is the most complex concerning the fats present in other foods considering that inside TAGs have more than 400 different fatty acids. The composition and the amount of milk fatty acids depend on the animal origin, stage of lactation, ruminal fermentation, the occurrence of mastitis, or feed-related factors. In fact, milk fatty acids are derivatives of the microbial activity in the rumen or feed. Fat fraction is composed of 70%, on average, of saturated fatty acids (SFAs) and 30% of unsaturated fatty acids. Inside SFAs, the largest quantity is represented by palmitic (30%) and then to follow myristic (11%) and stearic (12%). Short-chain fatty acids represent 11% of SFAs, in particular butyric (4.4%) and caproic (2.4%). Inside the unsaturated fatty acid fraction, there is oleic acid in concentrations within 24% to 35%, instead, polyunsaturated fatty acids represent around 2.3% of total fatty acids, with linoleic and α-linolenic acid accounting for 1.6% and 0.7%, respectively. Trans-fatty acids are also included in milk such as vaccenic acid (2.7%) and conjugated linoleic acid (0.34%–1.36%).

1.6 Vitamins in Milk

The vitamin fraction is divided into liposoluble vitamins A, E, and D and water-soluble vitamins. As regards to fat-soluble vitamins, their concentration inside milk is influenced by fat content, in fact,

low-fat and skim milk varieties have lower concentrations of A, E, and D vitamins. Vitamin A is very important in growth, immunity, development, and eye health. The content in milk of this vitamin depends, in particular, on fat amount, but it is also influenced by factors like season and animal feed. Whole milk is considered generally a good vitamin A source, supplying around 172 mg/100 g. In fat-free or skim milk there is an amount of vitamin A around 5 mg/100 g and 102 mg/100 g, respectively. For this reason, in many countries, it was chosen to fortify fat-reduced milk products to increase nutritional status and to reduce vitamin A deficiency, especially in affected children. Regarding vitamin D, milk itself doesn't have a considerable amount except when fortified. Previous studies have reported an amount within 5 and 35 IU/L, in accordance with reference nutritional tables. Commercial whole milk, to which vitamin D is added, presents an amount of around 40 to 51 IU/100 g. Recently, vitamin D attracted much more attention as a polyvalent micronutrient considering its protective actions. Studies have suggested vitamin D has anticarcinogenic, immunomodulatory, and cardioprotective effects and is crucial in calcium absorption, the bone mass formation, and can be a determinant to prevent osteoporosis. Regarding vitamin E, the concentration in milk is approximately 0.6 mg/L (USDA, 2007), but can increase 3-4 times with adequate feeding regimes. The recommended intake is 15 mg/day (Insel et al., 2004). Vitamin E is not a single compound; it includes tocopherols and tocotrienols. In whole milk, alpha-tocopherol is the main form of vitamin E (> 85%); gamma-tocopherol and alpha-tocotrienol are present to a lesser extent, about 4% each of the sum of tocopherols and tocotrienols (Kaushik et al., 2001). Some research indicates that high dietary vitamin E intake is associated with a reduced risk of cancer and coronary heart disease and that vitamin E can stimulate T lymphocytes and boost the immune system. Milk seems to have a role in promoting the absorption and transport of vitamin E from ingested food to chylomicrons (Hayes et al., 2001). Milk is important also for its richness in B complex vitamins, providing 10% to 15% of the daily recommended intake. These vitamins are also important enzymatic cofactors and participate in several metabolic pathways such as energy production from nutrients, hormones, and neurotransmitter synthesis. In particular, milk contains 50 μg of folate/L (USDA, 2007). Studies indicate that 5-methyl-tetrahydrofolate is the major form of folate in milk (Forssen et al., 2000). The recommended intake of folate is 400 μg/day for adults (Insel et al., 2004). Many scientists believe that folate deficiency is the most common of all vitamin deficiencies (Insel et al., 2004). It is generally accepted that folate supplementation (400 μg/day) before conception and during the first few weeks of pregnancy reduces the risk of neural tube defects. A recent study showed that higher total folate intake was associated with a reduced risk of incident hypertension, particularly in younger women (Forman et al., 2005). Furthermore, folate may play a protective role against coronary heart disease and some forms of cancer, but sufficient evidence is not yet available (Staff et al., 2005). The

complexity of folate metabolism suggests that several folate metabolites are involved in different reactions and that dihydrofolate and 5-methyl-tetrahydrofolate are the compounds active in inhibiting growth in colon cancer cells (Akoglu et al., 2001). Folate-binding proteins (FBP) are found in unprocessed milk, pasteurized milk, and skimmed milk powder, (Forssen et al., 2000) and several studies have suggested that FBPs improve the bioavailability of dietary folate as well as that the inclusion of cow's milk in the diet improves the bioavailability of dietary folate thanks to its FBP presence (Picciano et al., 2004). In a population-based study, consumption of milk and yoghurt was inversely associated with serum concentrations of total homocysteine and the authors explained this association with folate and riboflavin intake (Ganji et al., 2004). Milk is a good source also of riboflavin, 1.83 mg of riboflavin/L of milk. The recommended daily intake is 1.1 and 1.3 mg for women and men, respectively (Insel et al., 2004). Riboflavin is part of two important coenzymes that participate in numerous metabolic pathways in the cell. It plays a role in the antioxidant performance of glutathione peroxidase and in DNA repair via the ribonucleotide reductase pathway. Milk is also a good source of vitamin B12, being 4.4 μg/L (USDA, 2007). The daily recommendation is 2.4 μg (Insel et al., 2004). Vitamin B12 is found only in animal foods and plays a central role in the metabolism of folate and homocysteine by transferring methyl groups. Vitamin B12 deficiency can cause megaloblastic anemia and the damage of the myelin sheath.

1.7 Minerals Content in Milk

Milk has been naturally classified as an important calcium source but also many other elements are present in the milk mineral fraction. The average concentration of calcium is 1.2. μg/L of milk, divided between the aqueous and micellar phases. In the micellar phase, it is linked to caseins by phosphoseryl residues, in the aqueous phase, instead, calcium can be linked to whey proteins or to phosphate-forming salts. These phases are in thermodynamic equilibrium but if there are changes in the physicochemical milk conditions, such as temperature and pH, this situation can cause the passage of calcium from one phase to another. Besides calcium, milk is also a good source of phosphorus, which is present in the inorganic and organic forms. Organic phosphate is bound to organic molecules like proteins, organic acids, phospholipids and nucleotides, which are present in particular in the micellar phase; the inorganic form is represented by ionized phosphate, which depends by pH value and is inside the aqueous phase. As well as calcium, both forms are in equilibrium and their distribution may be affected by the pH. The concentration of phosphorus in milk, on average, is about 0.95 μg/L. Magnesium also can be found in milk in not very high concentration as well as in other dairy products. One liter of milk supplies 120 mg of magnesium, which represents 29% of the dietary

reference intake for this mineral. Milk is also a good source of microelements like selenium and zinc. One liter of milk supplies around 3/4 mg of zinc, which is present particularly inside the micellar phase associated with casein. Selenium is present in an average concentration of 30 mg/L, which represents around 67% of the dietary reference intake.

1.8 Sugars Content

Lactose is the main disaccharide present in milk and it is composed of glucose and galactose. It can be present in two isomeric forms, alfa (α) and beta (β) and in aqueous solution are in balance. It is hydrolyzed by lactase, a β-galactosidase, which has a special preference for the isoform β. This enzyme can be found in the small intestine mucosa membrane and after the hydrolysis of lactose, the two monosaccharides glucose and galactose are absorbed and transported to the liver through the portal vein and here the galactose is converted to glucose. In mammals, after weaning the β-galactosidase activity decreases significantly; this apparently doesn't happen in humans at the same grade. Its activity remains even during adulthood and for some reasons, intolerance symptoms occur when there is an enzymatic deficiency.

1.9 Milk Froth and Foaming

Cappuccino is a drink made with coffee and steamed milk. It usually consists of around 125 ml of milk and 25 ml of coffee. The foam and the cream must be good-looking, dense and in an amount equal to one third of the content in a cappuccino cup. Sometimes a sprinkling of cocoa or ground cinnamon is added. There are, in fact, several ways to prepare a cappuccino cup and recently, in order to make the preparation of cappuccino more appealing, modern techniques of art coffee or latte art have been developed by which the surface of the beverage is decorated with drawings made with a milk briquette or with manual tools (**Figure 8**).

Figure 8: An example of latte art.

The foam or cream of the cappuccino is normally prepared by blowing water vapour and air into the milk to get bubbles as small as possible, with a silky and smooth consistency.

The preparation of a perfect foam for cappuccino is an art but also the science could provide some guidelines for its preparation. For the formation of a good cream, it must be remembered that fats can destabilize the foam, the less fats are present in the milk and the easier it is to obtain a stable foam. With skimmed milk, usually 0.1% fat, it is therefore easy to have a persistent foam. It is less easy to have persistent foam using semi-skimmed milk and it is even more difficult if whole milk is used since it has a 3.6% of fat. However, it is preferable to use a whole milk that gives a tastier flavor, therefore, in this regard, a good compromise should be reached between taste and foam stability.

To have a good cream from a whole milk with 3.6% fat it is necessary to incorporate as much gas as possible. This is obtained starting from cold milk from the refrigerator (about 5 °C) because at these temperatures the air dissolves better in the liquid. Thanks to the steam injection, the temperature rises, and the lactoglobulins in the milk denature and change their structure, becoming an emulsifier that contributes to the stabilization of the bubbles. Furthermore, starting with cold milk, there is more time to form froth before the milk reaches a temperature of 65°C. Once the temperature has reached 65°C, the froth can be collected and added to the espresso coffee to make a cappuccino. A variant, however under discussion, for cappuccino preparation, is to heat the milk to 65 ° C with the steam injection, cool it and then blow in steam again, but there is the risk of excessively diluting the cappuccino (Ballarini, 2022).

Protein Role in Foam Formation

As discussed before, in cappuccino and cappuccino-like beverages, the characteristic of the foam determines the overall product quality, like their appearance, mouthfeel, texture, coffee aroma release rate, and the consumer satisfaction. Milk proteins are excellent foaming agents, but the foaming

properties of milk are also greatly affected by others factors like the protein content, casein micelle size, ratio of caseins to whey proteins, pH, proteolysis, minerals, presence of compounds with low molecular weight (like lipids and their hydrolyzed products) and compounds with an high molecular weight (polysaccharides); the characteristics of the foam is also affected by milk processing conditions (homogenization, aging and heat treatment), by method used to obtain the foam, and finally by the temperature. These factors can cause changes in the molecular structure, surface activity of the milk proteins and charge; or give interference and influence the stability of the foam competing with milk proteins in the formation of highly viscoelastic film. Some factors determine the foam stability while others affect the foamability.

Foam is an emulsion system where air bubbles are dispersed in the continuous phase. Normally, the atmospheric air is the dispersed gas, but in some cases, like in aerosol cream, inert gases (CO_2, N_2 or N_2O) are utilized. The continuous phase can be liquid or (semi-) solid and the different foams are classified into liquid-based or solid-based foams, respectively (Walstra, 1989). Cappuccino like-beverages, such as cappuccino, macchiato, and latte are typical products classified as liquid-based foams. These products differ for the coffee extraction ratio (espresso), milk, and foam (Hidden et al., 2012). The foam layer on the top is the critical element to obtain a high-quality product due to its light and soft texture, which is highly appreciated by consumers before the consumption, and for a smooth mouthfeel, tactile sensations, and creaminess during consumption. The desirable foam should have uniform and small (micro-size) size of air bubbles and should remain stable for at least 10 minutes during which half a cup of coffee is typically consumed (Ho et al., 2019; Xiong et al., 2020). In most coffee shops, the typical foam is prepared by steam injection into milk through very small openings or nozzles which are placed just below the milk surface. The steaming process is completed when the temperature of milk reaches approximately 60-65 °C. This is the ideal temperature for dispersing hot beverages like cappuccino-style drinks. During the foaming process, when the air is incorporated inside the bulk of the liquid, air bubbles are formed that can join together to minimize surface area, due to the high surface tension of the water in the system. However, the presence of suitable surfactants in the foaming system helps to stabilize the air bubbles as they quickly adsorb on the air-liquid interface to reduce surface tension and promote the formation of an elastic interfacial film surrounding the air bubbles (Kinsella, 1981). Milk proteins are excellent surfactant agents, due to their properties and distinctive structure (Kinsella & Morr 1984). Many studies have been conducted over the past few decades on milk frothing and many general principles are well known but the control of the milk foaming process to obtain a final quality product is still a difficult task for researchers and the dairy industry. Foam is a multi-phase, multi-component system and the foaming

process is determined by numerous factors, ranging from the source of the milk, the quality and composition of the milk to the processing conditions or even seasonality.

As discussed before, in cow's milk, the total protein content is around 3.5% (w /w) and the two main types of proteins are caseins and whey proteins and other minor groups of proteins such as those present in the milk fat globule membrane (Davoodi et al., 2016). Caseins represent approximately 80% of the total protein and comprise: αs-, β- and κ-caseins with an approximate weight ratio of 3:2:1. Caseins are disordered and flexible proteins, the irregular distribution of the residues of serine phosphate, acid and hydrophobic amino acids along the polypeptide chains gives them a very amphiphilic nature. These properties, together with the high content of randomly distributed proline residues, make caseins subjected to intermolecular interactions reversible via hydrophobic and ionic bonds, which is a function of pH, Ca^{2+} ion, and temperature. (Lee et al., 1992). Furthermore, due to the presence of cysteine groups, some caseins, such as αs2-casein, k-casein, could interact with other milk proteins through disulfide bonds. It was reported that among caseins, β-casein showed the greatest ability to reduce surface tension and produce foam, followed by αs-caseins and then k-casein (Lorient et al., 1989). However, casein β foam was the least stable compared to αs-caseins and k-foam due to the lack of tertiary structure and intermolecular interactions. Whey protein accounts for around 20% of the total protein content in milk and the most abundant are α-lactalbumin and β-lactoglobulin, which account for approximately 70-80% of the total whey protein. Most whey proteins exist as compact, globular proteins with their hydrophobic residues buried as far as possible within the molecules, are characterized by intermolecular disulfide bonds and are highly susceptible to heat-induced denaturation (Lee, Morr, and Ha 1992). With these characteristics, whey proteins are less effective than caseins in decreasing interfacial tension and subsequently foaming. When absorbed at the interface, whey proteins only partially unfold (retaining a considerable part of their secondary structure), resulting in the formation of viscous films to resist the collapse of air bubbles. Therefore, whey protein foam is typically more stable than casein foam.

General Mechanism of Foaming by Milk Proteins

As discussed above, immediately after the introduction of the gas, a gas-liquid interfacial film is formed, and its stability depends on the surface tension between the gas and the liquid. A decrease in surface tension facilitates the formation of the interfacial film, subsequently favoring the formation of foam (Zayas 1997). In milk frothing, proteins (casein or whey protein) play an important role in the initial formation and subsequent stabilization of the foam. As mentioned, milk proteins disperse

well in water and possess both hydrophilic and hydrophobic groups and the ability to reorient these groups at the air-water interface. These properties allow them to rapidly spread and adsorb on the interfacial regions during foaming, leading to a reduction in surface tension. In the interfacial regions, the proteins unfold and reorganize the polar and non-polar groups towards the aqueous and non-aqueous phases, respectively. This is followed by the interactions between the proteins unfolded mainly by electrostatic and hydrophobic interactions and hydrogen bonds, resulting in the formation of a strong, highly viscous, and highly elastic interfacial film that helps stabilize the air bubbles. (Huppertz, 2010; Zayas, 1997).

Influence of pH

Typically, the pH of fresh raw milk under normal conditions is around 6.5-6.6 and any change in pH results in a change in charge, structure (flexibility), surface activity, ionic strength, and ability to intra and intermolecular protein interaction (Ward et al., 1997) as well as the dissociation rate of minerals and caseins (Broyard & Gaucheron, 2015). All these alterations in turn affect the availability of surfactants, the rheological properties of the interfacial films and the viscosity of the milk, and therefore the foaming behavior.

Influence of the Foaming Temperature

The influence of the foaming temperature on the foaming behavior of whole and skimmed milk is very different because the foaming temperature affects the physical state of the fat globules present in whole milk. For whole milk, the foaming ability decreased dramatically over a temperature range of 5-35 °C and progressively increased with an increase in temperature from 45 to 85°C.

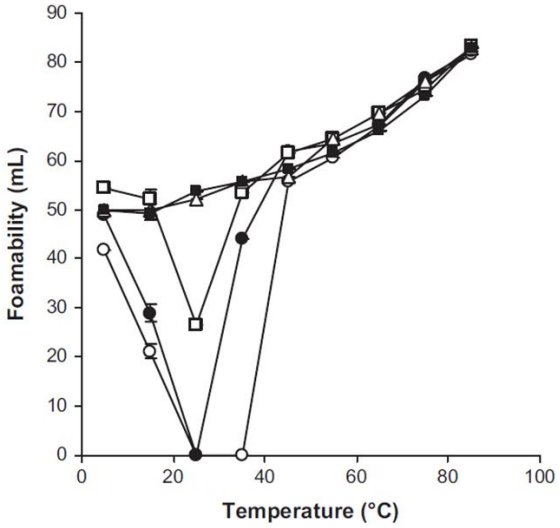


Figure 9: Foamability of cows' milk as a function of temperature (5–85°C): (○) raw whole milk, (●) pasteurized homogenized whole milk, (□) UHT homogenized whole milk, (■) UHT skim milk, (△) pasteurized skim milk (From: Kamath et al., 2008).

Likewise, stability of the foam produced by whole milk at temperatures below 40 °C was negligible, but it was significantly enhanced at temperatures above 40 °C. On the contrary, the capacity of the skimmed milk continuously increased with an increase of the temperature from 5 to 85 °C, with a maximum stability at 45 °C. For both whole and skimmed milk the foam produced at temperatures of 40-60 °C was much higher in capacity and stability than that produced at temperatures below 40°C (**Figure 9**). Similarly, for milk-based espresso coffees, an increase in the foaming temperature from 50 to 70 °C improved both the foam capacity and stability. However, foaming at very high temperatures (> 65 °C) causes a significant drop in foam stability (Kamath et al., 2008). It is probably due to excessive denaturation of proteins, particularly whey proteins, resulting in a reduction in the strength and viscoelasticity of the interfacial protein layers. Due to their poor thermal stability, at temperatures above 60 °C, whey proteins could be denatured and form polymers with themselves or with caseins (Qian et al., 2017).

Foaming Methods

By comparing the stability of the foam generated by steam injection, air bubbling and mechanical agitation, (Goh et al., 2009) found that the stability of whole milk foam was similar between the three

foaming methods. However, the effects of the foaming methods also depended on the types of milk. It was found that for raw whole milk at the same foaming temperature (65 °C), the mechanically blended foaming method exhibited much lower foaming than its steam and air injection counterparts. However, for raw skimmed milk and pasteurized whole and skimmed milk, the above-mentioned methods showed similar foaming ability (Ho et al., 2019). Furthermore, in one approach to foaming, such as steam injection, changes in process parameters, such as steam pressure and nozzle design, led to differences in foamability, foam stability and consistency, and size uniformity of air bubbles (Jimenez-Junca et al., 2015)

Importance of the Mineral Balance

Mineral salts have a significant impact on the conformation of proteins (caseins and whey proteins), on their stability and on their state of association and distribution between the colloidal and serum phase of milk through specific and non-specific interactions. (Augustin, 2000). In the previous sections of this thesis, it has been seen how the structure and stability of milk proteins have predominant roles in controlling the functional properties of milk and that the foaming properties are strongly determined by the types of proteins and their availability in the interfacial regions. Thus, the addition or removal of milk salts provides a means of manipulating the foaming properties of milk (Zayas 1997). The addition of NaCl affects the foaming ability of proteins due to the salt affecting the solubility, viscosity, unfolding, and aggregation of proteins. Frequently, NaCl increases foam overload and reduces foam stability. The effect of the different ions on foaming properties depends on their influence on the structure and proteins confounding. Multivalent metal ions improve the foam capacity of proteins (Zayas, 1997). NaCl added to soy protein suspensions increased foaming capacity, however, foam stability decreased. The increase in foam capacity was due to the higher protein solubility in the air/water interface during foaming. (Kinsella, 1976). However, the addition of NaCl delayed the partial denaturation of protein polypeptides required for protein-protein interaction and stability. It is due to the improvement of the foaming properties due to the salt to the reduction of the denaturation rate of proteins. The addition of NaCl can change protein conformation and solubility depending on the ions and proteins involved (Zayas, 1997).

Influence of the Proteolysis Process

Proteolysis is a breakdown of proteins into peptides and amino acids by proteolytic enzymes known as proteases. In terms of foaming, low molecular weight formed peptides diffuse to interfacial regions

to foam faster than proteins but compete and / or interfere with proteins in forming a highly viscoelastic film to stabilize the foam. Therefore, proteolysis of milk can improve foaming but reduce foam stability (van der Ven et al., 2002). Depending on the degree of protein hydrolysis (DH), which in turn has been determined by the hydrolysis conditions, many peptides with different properties and functionalities are produced. (Ho et al., 2021)

1.10 Interactions Between Milk Proteins and Coffee Compounds

The coffee is prepared by extracting the soluble material from the roasted grounds of the coffee beans in hot water (85-93 °C). Only in recent years coffee has been considered a functional food, due to its high phenolic content, mainly chlorogenic acids (CGA; they represent about 1-4% of the total dried composition of roasted coffee beans) (Farah, 2009). The phenolic compounds present in coffee are mainly the esters of hydroxycinnamic acids and quinic acid, the main compounds are caffeylquinic acids (CQA), dicaffeylquinic acids (diCQA) and feruloylquinic acids (FQA), each of which contains at least three isomers (Clifford et al., 2003). Several studies have reported that the phenolic compounds in coffee have various beneficial effects for human health; for example, antioxidant, antimicrobial, immunostimulating, hepatoprotective and hypoglycemic properties (Basnet et al. 1996; Nicolopoulos et al., 2020; Poole et al., 2019; Poole et al., 2017). Coffee is also known for activating the nervous system, improving perception and reducing fatigue, most of which are associated with caffeine (Sanchez-Gonzalez et al., 2005). However, some studies have suggested that these effects may be linked also with other coffee compounds that are responsible for the increase in blood pressure and the sympathetic nervous system activation (Corti et al., 2002; Klag et al., 2002). In light of this, numerous new coffee-based products have been developed in the market, furthermore, there has also been an increase in scientific studies concerning the compounds present in coffee and their effects on human health.

There are different methods to prepare a cup of coffee, and each one is based on a specific formulation that has become part of the consumption habits in different countries of the world.

In many world countries, coffee is consumed without adding milk or cream, in others, the addition of milk or cream is an essential part of making coffee-based beverages. Those include the addition of whole, semi-skimmed or skimmed milk and light or heavy cream, and also the use of steamed milk, condensed milk, plant milk, or frothy milk.

Milk can be added to a cup of espresso in various forms; steamed milk in caffe-latte (Fried, 1993), equal parts of milk and steamed milk froth in cappuccino (Castle & Nielsen, 1999), a spoonful of hot milk frothed in macchiato coffee (Miller, 2003), steamed hot milk (micro-foam) in flat white (to

enhance the flavor and create a velvety texture) (Kenneally, 2014), and many other recipes. Although the effect of adding milk on the bio-efficacy of caffeine is currently not completely known, several studies focused the attention on the addition of milk to coffee that could potentially decrease the antioxidant properties of coffee phenolic compounds, in particular CGAs.

From literature it is well known that the bioavailability and antioxidant properties of coffee polyphenols can be affected by possible interactions with milk components, particularly proteins (Al-Doghaither et al., 2017; Jeon et al., 2019; Quan et al., 2020). These interactions can significantly affect the sensory characteristics, digestibility, and bioavailability of the components of both coffee and milk (Liu et al., 2016). Several studies have confirmed that proteins such as whey proteins and caseins can bind to CGAs through both covalent and non-covalent interactions, suggesting that simultaneous consumption of coffee and milk may suppress effective absorption of CGAs in humans (Duarte & Farah 2011; Muralidhara & Prakash 1995). Although most of these studies reported an inhibitory (negative or masking) effect of milk on the antioxidant properties of CGAs (Al-Doghaither et al., 2017; Jeon et al., 2019; Quan et al., 2020), other studies reported a neutral effect (non-masking, without inhibition or enhancement) (Otemuyiwa et al., 2017; Renouf et al., 2010) or even a positive effect (enhancement) of milk on antioxidant activity and bioavailability of the GCP (Alongi et al., 2019). According to the data so far available in the literature, the effect of adding milk on the functionality of coffee depends on some factors such as the type of coffee, the composition of the milk, the ratio of milk to coffee, the temperature and the method of preparation of the beverage.

Several phenolic coffee compounds such as CGAs (caffeylquinic acids (CQAs), di-caffeylquinic acids and feruloylquinic acids), p-coumaric acid, caffeic acid, ferulic acid and proanthocyanidins, show antimicrobial, antiradical, hepatoprotective and immunostimulatory effects in humans (El-Messery et al., 2020; Quan et al., 2020; Duarte & Farah 2011; Niseteo et al., 2012). Once these compounds interact with milk proteins their healthy properties may be affected. Ali and coworkers (2013), found a concentration-dependent binding pattern of 5-CQA and various proteins and reported that the protein: 5-CQA ratio of 7:1 is the one where all the binding sites of proteins (independent of protein type) and 5-CQA are saturated and therefore interaction between them occurred. In particular, these authors found that the 5-CQA-β-lactoglobulin interaction is generated mainly due to their covalent interactions and thiol-disulfide interchanges. The protein-polyphenol affinity strongly depends on the polyphenol type, solubility, molecular free space, weight and the existence of glycosylated, hydroxylated and methylated functional groups (Yildirim-Elikoglu & Erdem 2018).

The presence and degree of these interactions between milk proteins and coffee phenolics depend on various factors such as the concentration of proteins and phenolics, the type of proteins and the structure of phenolics (Ozdal et al., 2013; Ozyurt & Otles, 2016). For example, structural

characteristics of phenolic acids such as type, methylation, hydroxylation, and steric hindrance have been reported to influence their binding affinity with β-casein (Li et al., 2020). In this study, out of six different phenolic compounds that were examined for their binding affinity with β-casein, caffeic acid was found to have the highest affinity, followed by CGA (Li et al. 2020). In addition there are other conditions such as temperature and pH that can influence the binding between coffee phenolic compounds and milk proteins forces leading to the alteration of bioactive potentials. On the other hand, it has also been reported that the ratio of proteins to phenolic compounds and the presence of other components (minerals) in the experimental matrix can influence the level of such interactions.

As explained above, the hydrophobic, covalent and non-covalent interactions that occur between milk proteins, fats, minerals, and coffee polyphenols show significant masking effects on the antioxidant activity of milk-coffee drinks (Dupas et al., 2006; Stojadinovic et al., 2013; Tagliazucchi et al., 2012) and can also affect the bio-accessibility and bioactivity of coffee phenolic compounds. In addition to proteins, the fats present in milk can interact with the phenolic compounds of coffee and have influence on their antioxidant activity, bio-accessibility and also on the caffeine content (Bhagat et al. 2019; Dupas et al., 2006; Tagliazucchi et al., 2012).

Some studies have reported the positive effects of milk components (both lipid and protein fraction) on the antioxidant properties and bioavailability of phenolic compounds in coffee. As discussed in the previous sections, complexations between milk components and polyphenols can form through hydrophobic or hydrophilic interactions and induce the formation of soluble or insoluble aggregates. Some research has shown that these complexes could enhance the antioxidant properties of the phenolic compounds in coffee (Bandyopadhyay et al., 2012).

It was shown that the interaction between milk lipids and coffee polyphenols could protect the latter during gastrointestinal digestion improving their bioavailability (Alongi et al., 2019; Jakobek, 2015; Ortega et al., 2009; Schramm et al. 2003). Other studies have also proved that complexes formed between phenolic compounds and milk proteins decrease the sensitivity of phenolic acid to degradation in the gastrointestinal tract, increasing their bioavailability.

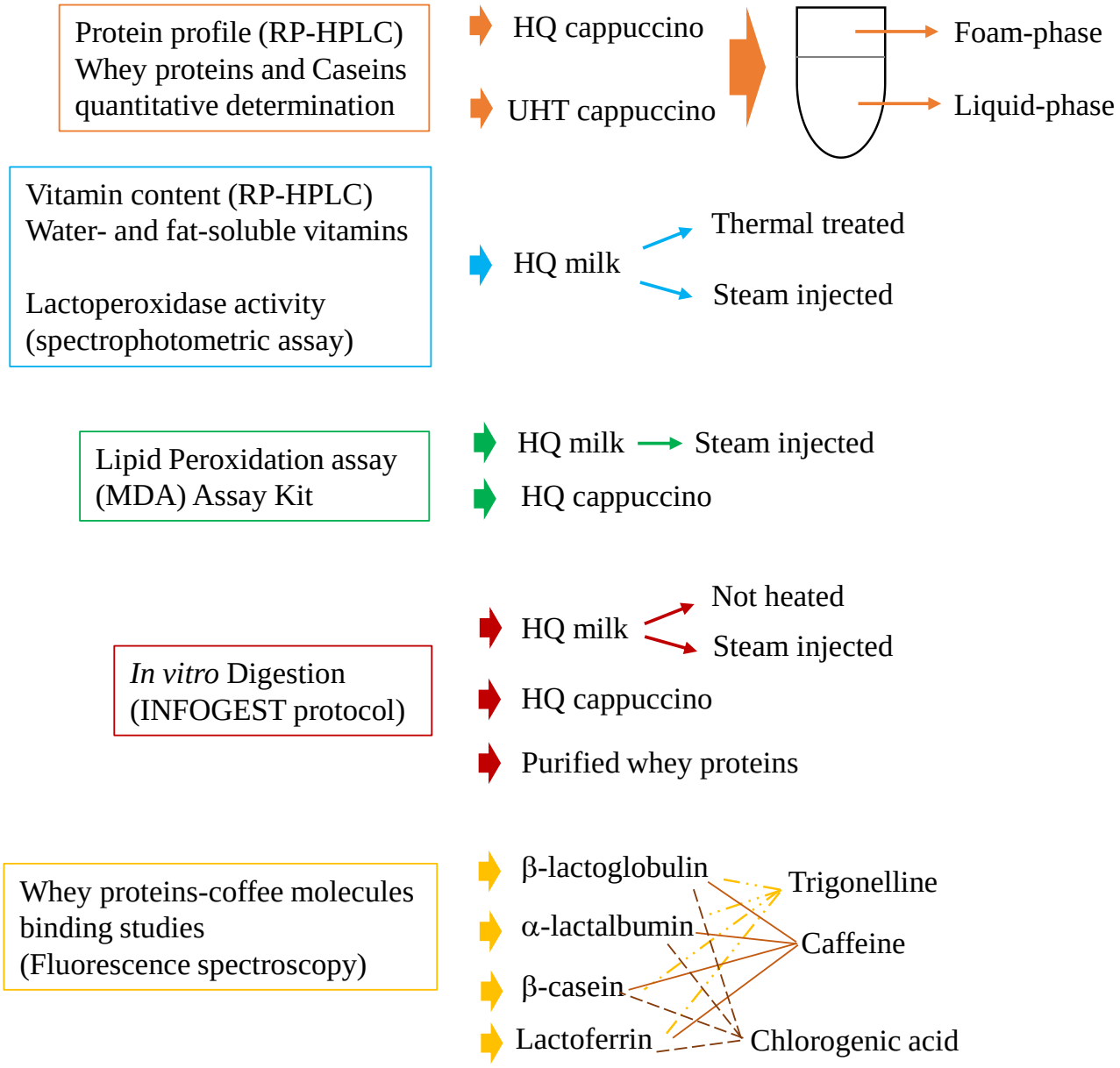
2. AIM OF THE RESEARCH PROJECT

This Eureka project aimed to characterize from the molecular, nutritional, and digestibility point of view samples of cappuccinos prepared using the Simonelli Group coffee machines under different steam injection conditions. The milk used in these experiments was the high-quality pasteurized milk (HQ, the type of milk most used by barmen for the preparation of cappuccino) and for some experiments was also used the UHT milk, for a comparison with the pasteurized milk.

For this purpose, the project had four main objectives, which are outlined below:

- Identification and quantification of the milk proteins involved in the formation of cappuccino froth, in particular those involved in the formation of the air-liquid interface. Since the quality of the milk is fundamental in the production of a high-quality cappuccino with a consistent and lasting foam, and the milk proteins are an important discriminant in high-quality milk, the knowledge of the type of proteins most involved in foam formation could help in choosing the right type of milk for this purpose.
- Characterization, from a nutritional point of view, of cappuccinos prepared under different conditions of injected steam and temperature, because nowadays the market requires milk/coffee-based beverages prepared at higher temperatures than those normally used for the cappuccino preparation, especially for some takeaway drinks.
- Determination of the binding affinity between the principal milk proteins (β-lactoglobulin, α-lactalbumin, β-casein, and lactoferrin) and some coffee compounds (caffeine, chlorogenic acid, and trigonelline) through fluorescence spectroscopy studies. The importance of this study lies in the fact that these complexes, once formed, could affect the palatability and the digestibility of the beverage, as well as the beneficial effect of the coffee compounds.
- Deepening the aspect concerning the digestibility of the cappuccino through *in vitro* digestibility studies, since in the literature are present very few works on this topic.

3. *EXPERIMENTAL DESIGN*



4. MATERIALS AND METHODS

4.1 Samples Preparation

The commercial milk types used to prepare cappuccino samples were pasteurized high-quality (HQ milk, nutritional value per 100 ml: fats 3.6 g; proteins 3.2 g; sugars 5.0 g), and ultra-high temperature milk (UHT milk, nutritional value per 100 ml: fats 3.6 g; proteins 3.1 g; sugars 4.8 g). The espresso blend used for cappuccino preparation was a 100% medium roasted Arabica coffee beans and consisted of beans from Brazil (Mundo Novo, natural), India (Plantation, S795 & Kent, fully washed) and Ethiopia (Yirgacheffe, Heirloom, fully washed). The equipment for milk heating, coffee beans grinding, and espresso preparation was obtained from the Nuova Simonelli SpA (Belforte del Chienti, Italy): Appia II semi-automatic two group espresso machines; Mythos Plus on demand espresso grinder with built in tamper. Each milk sample, initial volume of 150 ml and temperature of 8±1°C, was heated with the steam nozzle (steamer) of the coffee machine in a metal jug with a capacity of 350 ml. Steam at a gauge pressure of 110 kPa was injected into milk to reach temperatures ranging from 40° to 80°C. The quantity of water found in the sample after the steam injection treatment has been calculated and has been considered in the final calculation of the concentrations of the analytes under consideration. Furthermore, in all the analyses carried out in the cappuccino samples, the dilution made by the coffee (1.18-fold) has been also considered.

4.2 Protein Profile by Reversed Phase-High Performance Liquid Chromatography (RP-HPLC)

Protein profile analysis was performed on the liquid phase of cappuccinos prepared with HQ and UHT milk (named HQ cappuccino and UHT cappuccino), and on the respective foam phases (named HQ cappuccino foam and UHT cappuccino foam). Samples were prepared as described in the "Sample preparation" section heated by steam injection to reach a temperature range of 40-80°C. The HPLC system was an Agilent 1260 Infinity LC System (Agilent Technologies), consisting of a 1260 Infinity Quaternary Pump, 1260 Infinity Multisampler, 1260 Infinity Multicolumn Thermostat, and 1260 Infinity Diode Array Detector. Data were acquired by the OpenLab Software (Agilent technologies). The column was a 300Å C4 Prosphere (5 μm, 4.6 mm I.D., 150 mm, Alltech). Before being subjected to Reversed Phase-High Performance Liquid Chromatography (RP-HPLC), the foams were left at 4 ° C for about 2 hours to liquefy, subsequently each liquid phase of cappuccinos and respective foam phases samples were skimmed by centrifugation at 5000g for 30 min at 15°C. Each skimmed sample was then clarified by the addition of two volumes of CL buffer (0.1 M bis-tris, pH 8.0 containing 8 M urea, 1.3% trisodium citrate, 0.3% DTT), and centrifuged at 13000 g for 5 min

(Vincenzetti et al., 2008). One hundred microliters of clarified samples were loaded into the RP-HPLC column. The column was equilibrated in trifluoroacetic acid (TFA)/H2O 1:1000 v/v (buffer A) and elution was achieved by the following step gradient with TFA/H2O/acetonitrile 1:100:900 v/v (buffer B): %B = 0, time = 10 min; %B = 20, time = 10 min; %B = 40, time = 0.1 min; %B = 60, time = 40 min. The flow rate was 1 ml/min and fractions of 0.5 ml were collected. The proteins eluted from RP-HPLC columns were monitored at 280 nm. Each standard solution of bovine milk β-lactoglobulin (from 0.033 to 0.33 mg/ml), bovine milk α-lactalbumin (from 0.033 to 0.33 mg/ml), human lactoferrin (from 0.005 to 0.2 mg/ml), bovine β-casein (from 0.2 to 1.0 mg/ml) and bovine αs1-casein (from 0.024 to 0.4 mg/ml), was prepared in CL buffer. A 100-µl solution of each standard was separately loaded on the RP-HPLC column. The area of each standard peak was measured using the valley-to-valley integration mode and quantification was achieved by a calibration curve obtained relating the concentration in micrograms of each standard loaded in the column to the peak area corresponding to each concentration. From each milk sample the quantity of lysozyme, β-lactoglobulin, α-lactalbumin, lactoferrin, β-casein and αs1-casein was determined by using the calibration curve. Furthermore, standard solutions of coffee compounds such as chlorogenic acid, trigonelline and caffeine (Merck-Sigma Aldrich, Milano) each prepared at the concentration of 0.1 mg/ml was separately load on RP-HPLC and used for the identification of the coffee compounds in the cappuccino preparations.

All measurements were conducted in triplicate and the results averaged and the standard deviations calculated.

4.3 In-Gel Protein Digestion

After RP-HPLC, each eluted peak was loaded to a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) performed as described by Laemmli (1970), under reducing conditions (see "*Other analytical procedure*" section). The resulting protein bands were thus subjected to the in-gel digestion of proteins for the subsequent mass spectrometry analysis. In-gel digestion was performed following the protocol of Shevchenko and co-workers (2006). The spots were excised from the gel and cut into small pieces (ca. 1 x 1 mm), destained with 100 µl of 100 mM ammonium bicarbonate/acetonitrile (1:1, v/v), incubated for 30 min and subsequently treated with 500 µl of neat acetonitrile until pieces became white. After acetonitrile removal, the in-gel digestion was performed incubating the gel pieces with trypsin buffer (13 µg/ml trypsin in 10 mM ammonium bicarbonate containing 10% (v/v) acetonitrile) for 30 min on ice to allow the trypsin to diffuse into the gel.

Subsequently, 10-20 µl of 100 mM ammonium bicarbonate was added to cover the gel and the samples were then incubated overnight at 37 °C. The peptide digestion products were extracted adding 100 µl of extraction buffer (1:2 (v/v) 5% formic acid/acetonitrile) to the tube followed by incubation for 15 min at 37 °C in a shaker. The combined extracts were recovered and dried in a vacuum concentrator at room temperature. A piece of blank gel, without spots, and a standard protein band from a commercial bovine serum albumin were submitted to the same procedure, and used as negative and positive controls, respectively. The samples were then subjected to LC-MS/MS analysis. These experiments were performed in collaboration with Dr Massimo Ricciutelli (HPLC-MS Laboratory, University of Camerino)

4.4 LC-MS/MS Analysis

After the digestion, the tryptic peptides were dissolved in 100 µL of 0.1% (v/v) trifluoroacetic acid and subjected to a reversed phase chromatography (C18 Gemini-NX, ml particle size, 110 Å pore size, 250x 4.6 mm, Phenomenex, Torrance, CA.) connected to a HPLC Agilent Technologies 1100 Series (Agilent Technologies, Santa Clara, CA.). The column effluent was analyzed by MS using an electrospray ion trap mass spectrometer (Agilent Technologies LC/MSD Trap SL) operating in positive ion mode over the mass range 300-2200 amu (atomic mass units). MS spray voltage was 3.5 kV, and the capillary temperature was maintained at 300 °C. Obtained spectra were extracted and analysed by the MASCOT software (www. matrixscience.com) with the following search parameters: database, NCBInr taxonomy: *Bos taurus*; enzyme, trypsin; peptide tolerance, 1.2 Da; MS/MS tolerance, 0.6 Da and allowance of one missed cleavage.

4.5 Water-Soluble Vitamin Content

Water-soluble vitamin content has been determined on HQ milk samples heated with the heater digital Thermoregulator (Velp Scientifica, Usmate Velate MB) or heated with the steam nozzle (steamer) of the coffee machine Appia II semi-automatic (Nuova Simonelli SpA, Belforte del Chienti, Italy), for 20-60 seconds in the temperatures range of 50-80°C. During preparation, the samples were protected from light by wrapping tubes and flasks with aluminum foil. Before being analyzed for the vitamin content, the milk samples were processed according to the method described by Albalá-Hurtado and co-workers [1997 A]. The vitamin extraction procedure from milk samples was performed in a darkened room. Briefly, to 5 ml of sample, 0.5 g of TCA was added, and the mixture was stirred for 10 min over a magnetic stirring plate. Subsequently, the mixture was centrifuged for 10 min at 1250

g, and the first supernatant was recovered. To the precipitate, 3 mL of 4% TCA was added, mixed for 10 min, and then centrifuged again (same conditions as above). After centrifugation, the supernatant was recovered and combined with the first supernatant. These two acid extracts were brought to 10 mL with 4% TCA and filtered through a 0.45 μm filter (Whatman, Maidstone, UK), before being subjected to the RP-HPLC analysis. The chromatographic courses were performed according to Albalá-Hurtado and co-workers (1997 a), using the same HPLC system described in the previous section. The column was a C18 HiQSil HS, 5 μm, 4.6 mm i.d, 250 mm (Kya Tech Corporation, Tokyo, Japan). The mobile phase was methanol (85:15), containing 0.5% triethylamine, 2.4% glacial acetic acid, and 5 mM octane sulfonic acid (pH 3.6). The analyses were carried out isocratically at 25 °C, with a flow rate of 0.9 mL/min; the total run was 40 min. The injection volume was 100 μL. The eluent was detected at 254 and 270 nm.

Standard solutions of vitamin B1 (from 0.1 to 18.7 μM), vitamin B2 (from 0.5 to 15 μM), nicotinamide (from to 7.5 μM), nicotinic acid (from 0.75 to 20.0 μM), vitamin B6 (from 0.75 to 20.0 μM) and folic acid (from 0.3 to 7.5 μM) were prepared in ultra-pure water. A 100 μL solution of each standard was separately loaded on the RP-HPLC column. The area of each standard peak was measured using the valley-to-valley integration mode, and quantification was achieved by a calibration curve obtained relating the concentration (μM) of each standard loaded in the column to the respective peak area. The quantity of vitamin B1, B2, nicotinamide, nicotinic acid, vitamin B6, folic acid in HQ milk was determined using the calibration curve. The vitamins in the milk samples were identified by comparison of their retention times and UV-spectra with those of the respective vitamin standards.

4.6 Fat-Soluble Vitamin Content

Also in this case, fat-soluble vitamin content has been determined on HQ milk samples heated with the heater digital Thermoregulator (Velp Scientifica, Usmate Velate MB) or heated with the steam nozzle (steamer) of the coffee machine Appia II semi-automatic (Nuova Simonelli SpA, Belforte del Chienti, Italy), for 20-60 seconds in the temperatures range of 50-80°C. The method for fat-soluble vitamin content determination, was based on the protocol of Albalá-Hurtado and co-workers with some modifications [1997 B]. Before performing the analysis, milk was prepared by the following protocol: to 5 ml of milk in a graduated flask, 0.1 g of ascorbic acid was added followed by the addition of 10 ml of absolute ethanol and 2 ml of 60% potassium hydroxide solution. The graduated flask was closed hermetically and kept it in the dark, the mixture was incubated at 60 °C for 30 min under shaking, cooled for 5 min in cold water (water and ice) and 5 mL of n-hexane, was added

followed by vigorous shaking for one minute. The content was transferred into a 50 mL separatory funnel and the phase was left to separate for 5 minutes; this step was repeated three times. All organic phases (transparent upper part after separation in the funnel are combined. The collected solvent (approximately 15 ml) was evaporated by a Speed-Vac evaporator.

The standard stock solutions of the vitamins: retinol (vitamin A) (500 μg/mL), cholecalciferol (vitamin D3) (100 μg/mL) and α-tocopherol (vitamin E) (500 μg/mL) were prepared by weighing accurately 5, 10 and 50 mg of vitamins A, D3 and E in volumetric flasks of 10, 100 and 100 ml respectively. Sequentially, a volume of 2, 5, and 5 mL of absolute ethanol was added to each flask to aid solvation, and each flask was filled to the mark using methanol (HPLC PLUS Gradient grade, Carlo Erba reagents, Cornaredo, MI). 10mg/ml of vitamin K1 stock solution was prepared dissolving the vitamin in methanol.

These stock solutions were stored at -20 ° C for a period not exceeding one week for vitamins A and D3 and two weeks for vitamin E. All standards were protected from light during storage to minimize degradation.

Standard solutions of vitamin A (from 0.05 to 10 μg/mL), vitamin D3 (from 0.05 to 10 μg/mL), vitamin E (from 0.05 to 50.0 μg/mL) and vitamin K1 (from 0.1 to 100 μg/mL) were prepared from the respective standard stock solution.

The RP-HPLC analyses were performed according to Albalá-Hurtado and co-workers [1997 B], using the same HPLC system and the same column described for the water-soluble vitamin content. The optimized mobile phase was an isocratic mixture of methanol and water (95: 5, v/v) with a flow rate of 1 mL / min, monitoring at 280 nm (optimal absorbance for the detection of vitamins A, E and D3, simultaneously) and a total analysis time of 15 min. The vitamins in the milk samples were identified by comparison of their retention times and UV-spectra with those of the respective vitamin standards.

4.7 Lactoperoxidase Activity

Lactoperoxidase activity was evaluated by a continuous spectrophotometric rate determination using as substrate 2,2'-Azinobis (3-Ethylbenzthiazoline-6-Sulfonic Acid) (ABTS, Sigma Chemical Co.) following a protocol described by Pruitt and Kamau (1994). In this method, reduced ABTS is oxidized by H_2O_2 to form oxidized ABTS and H_2O. The reaction mixture contained 75 mM potassium phosphate pH5.5, 23 mM ABST and 0.0008% (w/w) hydrogen peroxide and was started by the addition of 10μl of each milk sample diluted 1:10 with the reaction buffer. The sample consisted of HQ milk heated for 20 seconds with the heater digital Thermoregulator (Velp Scientifica, Usmate Velate MB) or with the steam nozzle (steamer) of the coffee machine Appia II semi-automatic (Nuova

Simonelli SpA, Belforte del Chienti, Italy), in the temperatures range of 50-80°C. Absorbance measurement was made at λ=436nm in continuous after 5 min reaction. One unit of lactoperoxidase is defined as the amount of the enzyme that oxidizes 1.0 μmol of ABST per minute at pH 5.5 and 25°C. For the calculation of the enzymatic activity the **equation 1** was used:

Equation1

$$U/ml = \frac{(\Delta A436nm/TEST - \Delta A436nm/BLANK)\ x\ TOT\ ml}{29.3\ x\ Enzyme\ ml}$$

were A_{436nm}/min blank being the absorbance of 75 mM potassium phosphate pH5.5 with the substrate and the hydrogen peroxide but in absence of milk; 29.3 is the millimolar extinction coefficient of oxidized ABTS at 436nm.

4.8 Hydrogen Peroxide Determination in Freshly Brewed Espresso

Hydrogen peroxide (H_2O_2) concentration in freshly brewed espresso has been determined by using a spectrophotometric method based on the peroxidase-catalyzed oxidation of 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) to form the stable green radical $ABTS^{\bullet+}$ that can be evaluated at 436 nm. This method is based on a modification of the protocol used for the evaluation of the lactoperoxidase assay previously described with the difference that the freshly brewed coffee is used as a substrate to evaluate the concentration of H_2O_2. The espresso blend used in these experiments has been described above in the section "*Sample preparation*". The reaction mixture consisted of 75 mM potassium phosphate buffer (PPB) pH5.5, 23 mM ABST and freshly brewed espresso diluted 1:1 with ultrapure water. The reaction was started by the addition of 0.1 μg (25 mU) of peroxidase (from horseradish, Type VI, Sigma Chemical Co.). The absorbance increase at 436 nm is followed for 5 min. For the calculation of the enzymatic activity, the above-mentioned **equation 1** was used. At the same time the peroxidase activity was evaluated in presence of different standard H_2O_2 concentrations (0.086, 0.144, 0.17, 0.24 and 0.29 mM) to allow the calculation of the K_m and the V_{max} of the peroxidase for H_2O_2 through the Lineweaver-Burke equation. These kinetic parameters were subsequently used to calculate the concentration of hydrogen peroxide present in the espresso through the Michaelis-Menten **equation 2**:

Michaelis-Menten ***equation 2***

$$v = \frac{(V_{\max} \times [S])}{(K_m + [S])}$$

where:

v is the velocity, the activity (U/ml) of peroxidase calculated using as substrate different amounts (µl) of freshly brewed espresso dilute 1:1.

V_{max} is maximum velocity when all the enzyme is complexed to the substrate, [S] is substrate and K_m is the Michaelis–Menten constant.

In a recent article, Cai and co-workers (2018) proposed a new spectrophotometric method for the measurement of hydrogen peroxide in aqueous solutions exploiting the peroxidase-catalyzed oxidation of ABTS. In this method the generated ABTS·+ was evaluated at 415 nm, with different H_2O_2 concentrations. In this work, the H_2O_2 concentration in a freshly brewed coffee has been evaluated also considering the method proposed by Cai and co-workers but with some modifications. First of all, the reaction mixture is the same as that proposed previously (75 mM PPB pH5.5; 23 mM ABST; espresso diluted 1:1 with ultrapure water). The reaction was started by the addition of 0.1 µg (25 mU) of peroxidase (from horseradish, Type VI, Sigma Chemical Co.). The change in absorbance at 436 nm (ΔA_{436}) was evaluated after 0.5 minutes and the corresponding blank value was subtracted (75 mM PPB pH5.5; 23 mM ABST; the peroxidase but without the diluted espresso sample). A calibration line was generated by relating the ΔA_{436} subtracted with the blank, versus increasing concentrations of standard H_2O_2 solutions. The concentration of H_2O_2 in the freshly brewed espresso was calculated through the **equation 3**:

Equation 3

$$[H_2O_2] = \frac{\Delta A * V}{\gamma * \varepsilon * v}$$

where:

$\Delta A = \Delta A_{436}$ subtracted with the blank;

V= final volume of the reaction mixture

γ = stoichiometric factor of ABTS·+ generation which is calculated by dividing the slope of the calibration curve by the millimolar extinction coefficient of oxidized ABTS at 436 nm that is 29.3;

ε = 29.3 (millimolar extinction coefficient of oxidized ABTS at 436 nm)

v= volume of the sample

4.9 Lipid Peroxidation Assay (TBARS Assay)

Lipid peroxidation assay has been performed on HQ milk and HQ cappuccino sample both treated with the steam injection at the temperature of 60, 70 and 80°C.

Lipid peroxidation is the degradation of lipids that occurs because of oxidative damage and is a useful marker for oxidative stress. Polyunsaturated lipids are susceptible to an oxidative attack, typically by reactive oxygen species, resulting in a well-defined chain reaction with the production of end products such as malondialdehyde (MDA). In this work the MDA concentration has been assessed by the Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich, St. Louis, MO) following the indications provided by the datasheet. Lipid peroxidation is determined by the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form a colorimetric product, proportional to the MDA present, that can be evaluated at λ= 532nm by a spectrophotometer. All samples and standards were run in triplicate. Ultrapure water has been used for the preparation of all standards and samples. MDA standard concentration was 0.02, 0.04, 0.06, 0.08, 0.1 mM. Before performing the assay, milk samples were prepared as follows: to 150 µl of milk, 3 µl of Butylhydroxytoluene (BHT) and 150 µl of 10% trichloroacetic acid (TCA) were added and the mixture was centrifuged at 13000g for 5 min in order to remove the proteins. 200 µl of supernatant was directly used for the MDA assay strictly following the protocol described in the kit datasheet.

4.10 In-Vitro Digestion

According to the INFOGEST protocol described in the literature, on the static *in vitro* simulation of gastrointestinal food digestion (Brodkorb et al., 2019), an *in vitro* digestion protocol was developed to test the digestibility of HQ milk, HQ cappuccino and some purified milk proteins. HQ cappuccino was prepared as described in the "*Sample preparation*" section, using the espresso coffee machine. The purified milk proteins subjected to the digestion protocol were β-casein (β-CN), β-lactoglobulin (β-LG) and α-lactalbumin (α-LG).

The digestion protocol involves three stages: preparation, sample digestion, and treatment procedure with subsequent analysis. For the preparation of the digestion *in vitro*, the activities of all digestive

enzymes and the concentrations of bile salts should be experimentally determined, using the recommended standardized assays for amylase, pepsin, and lipase (both gastric and pancreatic), trypsin and chymotrypsin. This first stage of preparation is the most important since failure to properly dose enzyme activity will lead to incorrect digestion rates of components, potentially changing the overall digestion of the food. In the digestion procedure, the food is exposed to three successive digestive stages: oral, gastric and intestinal. For *in vitro* static digestion methods, the experimental conditions are constant throughout each step. In the **Table 3** are shown the buffer used during the different stages of the *in vitro* digestion. In the oral phase the food is diluted 1:1 (wt/wt) with the simulated salivary fluid (**SSF**). In these experiments, 5 ml of HQ milk, or HQ cappuccino or each solution containing the standard milk protein were mixed with 5 ml of SSF at pH 7.0, without salivary amylase. The mixture was incubated for 2 minutes. The oral bolus is then diluted 1:1 (vol/vol) with the simulated gastric fluid (**SGF**) at pH 3.0 and gastric enzymes (pepsin and gastric lipase) and incubated under stirring for two hours. The obtained gastric chyme is diluted 1:1 (vol/vol) with simulated intestinal fluid (**SIF**) at pH 7, bile salts and pancreatic enzymes (pancreatin based on trypsin activity or as single enzymes) and incubated for a further two hours.

Table 3: regarding the buffers used for the different stages of digestion, with the specific quantities of electrolyte stock solutions added.

Volumes of electrolyte stock solutions of digestion fluids diluted with water (final volume 400 ml) (1.25×)						
	Simulated salivary fluid **SSF (pH 7)**	Final salt concentration in SSF	Simulated gastric fluid **SGF (pH 3)**	Final salt concentration in SGF	Simulated intestinal fluid **SIF (pH 7)**	Final salt concentration in SIF
Salt stock solution (SSS) concentration	ml of SSS	mM	ml of SSS	mM	ml of SSS	mM
KCl 0.5 M	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4 0.5 M	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$[a] 1.0 M	6.8	13.6	12.5	25	42.5	85
NaCl 2.0 M	-	-	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$ 0.15 M	0.5	0.15	0.4	0.12	1.1	0.33
$(NH_4)_2CO_3$[*] 0.5 M	0.06	0.06	0.5	0.5	-	-
HCl 6.0 M	0.09	1.1	1.3	15.6	0.7	8.4
$CaCl_2(H_2O)_2$[b]0. 3.0 M	0.025	1.5	0.005	0.15	0.04	0.6

[a] The use of carbonate salts in the electrolyte solutions requires the use of sealed containers with limited headspace. $NaHCO_3$ salts were replaced by NaCl in the electrolyte solutions to avoid possible pH variations induced by the degassing of CO_2.

[b]: $CaCl_2(H_2O)_2$ should be added immediately before use. Volumes in Table 2 are indicated for a typical experiment of 5 mL of SSF.

Regarding standard proteins used for the experiment, the quantities normally present in milk were replicated, as reported in the literature, then dissolved in 5 ml of ultrapure water. The concentration used for digestion of standard β-CN was equal to 9-11 mg/ml, then 2-4 mg/ml for the β-LG and 0.6-1.7 mg/ml for α-LA.

Digestion procedure

In-vitro digestion is divided into several steps the samples have undergone and they are listed in detail below:

- **Oral phase** (2 min). Dilution of 5 ml of sample with SSF at a ratio of 1:1 (wt/wt) to achieve a swallowable bolus with a paste-like consistency. Salivary amylase is not necessary in this case because it is needed only to digest starch-containing food.
- Addition of $CaCl_2(H_2O)_2$ in order to achieve a total concentration of 1.5 mM in SSF
- Incubation while mixing with a shaking incubator for 2 min at 37 °C.

- **Gastric phase** (2–3 hours). Pre-warming of the SGF electrolyte stock solution at 37°C. Addition SGF electrolyte stock solution to the oral bolus to achieve a final ratio of 1:1 (vol/vol) and then adjustment of the pH to 3.0 by adding a defined volume of HCl previously determined during a pH-test adjustment experiment.
- Addition of $CaCl_2(H_2O)_2$ solution in order to achieve a final concentration of 0.15 mM in SGF.
- Addition of the porcine pepsin (Sigma, cat. no. P7012) solution prepared in water to achieve an activity of 2,000 U/mL in the final digestion mixture.
- Addition of the gastric lipase (Sigma, cat. no. L3126-25G) solution prepared in water to achieve an activity of 60 U/mL in the final digestion mixture.
- Addition of water in order to achieve a 1× concentration of SGF.

- Incubation of the samples at 37 °C, mixing the digestive mixture with the shaking incubator for 2 hours from the point at which pepsin was added.
- Stop the reaction by adding a 1.0 M of Na_2CO_3 solution to raise the pH 7 and block pepsin activity.

- **Intestinal phase** (2–3 h). Pre-warming of the SIF electrolyte stock solution in a 37 °C water bath.
- Addition of SIF electrolyte to the gastric chyme to achieve a final ratio of 1:1 (vol/vol).
- Adjustment of the pH to 7.0 by adding a defined volume of NaOH previously determined during a pH-test adjustment experiment.
- Addition of $CaCl_2(H_2O)_2$ solution in order to reach a concentration of 0.6 mM in SIF.
- Addition of porcine pancreatin (Sigma Aldrich, cat. no. P7545-500G) suspension to achieve a trypsin activity of 100 U/mL in the final mixture and then addition of water in order to achieve a 1× concentration of the SIF.
- Incubation of the samples at 37 °C, using a shaking incubator to mix the digestive mixture sufficiently for 2 h, starting at the point when pancreatic enzymes were added.
- Stop the reaction by adding a 5 mM Pefabloc SC (4-(2- aminoethyl) benzenesulfonyl fluoride hydrochloride) solution.

In vitro digestion of individual milk proteins

To fine-tune the protocol using different concentrations of digestive enzymes and to understand the behaviour of the individual main proteins present in bovine milk, β-lactoglobulin (Sigma-Aldrich, cat. No. L3908-1G), β-casein (Sigma Aldrich, cat. no. C6905-250MG) and α-lactalbumin (Sigma Aldrich, cat. no. L6010-100MG), and bovine serum albumin were subjected to *in vitro* digestion in the amount of 2-4 mg/ml, 9-11 mg/ml, 0.6-1.7 mg/ml, and 0.4 mg/ml, respectively to replicate the quantities normally present in bovine milk.

The total digestion of the β-lactoglobulin started from the gastric phase lasting 2 hours. At intervals of 30 minutes, 100 µL of the sample solution was withdrawn, and the reaction was stopped by adding Na_2CO_3 1 M in order to reach pH 7.0. The same procedure was carried out during the intestinal phase, also lasting 2 hours and with the withdrawal of a 100 µL solution sample at intervals of 30 minutes, stopping the reaction using 5 mM Pefabloc SC (pancreatin inhibitor). A standard solution of β-lactoglobulin was used also as blank, subjected to the same procedure but without enzymes. All

aliquots withdrawn during the gastric and intestinal phase were subsequently subjected to electrophoretic analysis or to the OPA assay (described in the next section). The total digestion of the α-lactalbumin, albumin, and β-casein was carried out using the same procedure described for the β-lactoglobulin. Also in this case, a standard solution of each protein was subjected to the same digestion process without using the digestive enzymes and was considered as a blank. The quantity of pancreatin in the intestinal phase, that was assessed to 0.07 mg/ml.

The scheme below summarizes the protocol used in the digestion of individual milk proteins.

Gastric digestion

1. SGF preparation: mix pepsin (10mg) and lipase (0.5mg) in 1 ml of SGF buffer
2. Adjust the solution to pH 3 with the addition of HCL 1M containing 0.15 mM of $CaCl_2$
3. Preparation of the incubation mixture:

- 1 ml of SGF containing the pepsin + lipase mix
- 5 ml of the sample (for each milk protein, at the same concentration present in the milk)
- 4 ml of SGF → Final volume: 10 ml

INCUBATE AT 37°C

5. Stop reaction with the addition of 40 µl of Na_2CO_3
6. At the end of the gastric phase, adjust to pH 7.0 with the addition of NaOH 1M

Intestinal digestion

7. Preparation of the pancreatin solution (0.07 mg/ml in the SIF buffer)
8. Preparation of the incubation mixture:

- 10 ml solution coming from the gastric digestion
- 9 ml of SIF buffer
- 1 ml of pancreatin solution + $CaCl_2$ 0.6 mM

INCUBATE AT 37°C

9. Stop reaction by adding di 10 µl di Pefabloc

In vitro digestion of HQ milk and HQ cappuccino samples

The protocol for the in *vitro* gastrointestinal digestion of HQ milk and HQ cappuccino, was similar to that used for the digestion of the individual milk proteins shown in the previous scheme. The samples consisted of HQ milk (not treated with the steam injection), and HQ cappuccino (prepared at 60°C with the coffee machine). 5 ml of each sample was used in these experiments (total protein amount: 0.23g). It was necessary to adjust the pancreatin concentration used in the intestinal phase in order to obtain a complete digestion of the HQ milk and HQ cappuccino samples. In this regard, different concentrations of pancreatin were tested in order to have for both milk samples a gradual and complete digestion. As a result, it was found an optimal amount of porcine pancreatin of 0.2 mg/ml for the intestinal phase digestion. All the other parameters remained unchanged with respect to the scheme shown previously

4.11 Analysis of the Digested Milk Proteins (OPA Assay)

The analysis of the digested milk proteins was performed using an SDS-PAGE, performed as described by Laemmli (1970), under reducing conditions (see "*Other analytical procedure*" section). In addition to the analysis of the samples by SDS-PAGE the *in vitro* digestion products were analyzed by the O-phthalaldehyde assay (OPA assay), based on amino groups released during proteolysis of a protein substrate.

This method is based on the degree of hydrolysis (DH) of proteins quantified by determination of the free amino groups released during the hydrolysis process by using compounds which react specifically with amino groups, such as O-phthaladehyde (OPA). The OPA method is based on the specific reaction between OPA and primary amino groups, in the presence of a thiol to form 1-alkylthio-2-alkyl-substituted isoindoles (Medina Hernandez et al., 1990a, b). The isoindoles formed can be quantified spectrophotometrically at 340 nm or fluorometrically at 455 nm. In this experiment, the DH was calculated using the OPA method described by Spellman et al., (2003) with some modification. The samples subjected to gastrointestinal digestion and subsequent OPA assay were: HQ milk (not treated with the steam injection), HQ milk 60°C (HQ milk heated with by stem injection up to a temperature of 60°C, and HQ cappuccino (prepared at 60°C). Gastrointestinal digestion was performed in these three samples as described in the previous section (*In vitro digestion of HQ milk and HQ cappuccino samples.*

The OPA/NAC (N-acetyl-cysteine) reagent (100 mL) was prepared by combining 10 mL of 50mM OPA (in methanol) and 10 mL of NAC 50 mM, 5 mL of 20% (w/v) SDS, and 75 mL of borate buffer (0.1 M, pH 9.5). The reagent was covered with aluminum foil to protect from light and allowed to stir for at least 1 h before use. The OPA assay was carried out by the addition of 20 μL of sample (the digestion product or a standard) to 2.4 mL of OPA/NAC reagent. Before the analysis, the samples were centrifuged at room temperature for 10 minutes at 1000 rpm. The absorbance of this solution was measured at 340 nm with SHIMADZU UV-2450, UV–Vis-spectrophotometer (SHIMADZU CORPORATION, Kyoto, Japan) against a control cell containing the reagent and 20 μL of the buffer in which sample is dissolved. The intrinsic absorbance of the sample was measured before OPA addition and subtracted. A standard curve was prepared using l-isoleucine (0–2 mg/mL).

DH values were calculated using the following **equation 4**:

$$DH\% = \frac{100n}{N}$$

where *n* is the average number of peptide bonds hydrolyzed, N the total number of peptide bonds per protein molecule.

It can be shown by the **equation 5** that:

$$n = \frac{\Delta AbsMd}{\varepsilon c}$$

Where ΔAbs is the Abs of test sample at 340 nm - Abs unhydrolysed sample at 340 nm, M the molecular mass of the test protein (Da), d the dilution factor e the molar extinction coefficient at 340 nm (6000 mol^{-1} cm^{-1}) (Church et al., 1985), and c the protein concentration (g L^{-1}). Values were calculated taking the approximate protein content present inside bovine milk to be 80% caseins, 17% α-lactalbumin, 12.5% β-lactoglobulin and 1% bovine serum albumin. Using these values the average molecular mass of the proteins in bovine milk was calculated to be 25770 Da (M), with an average of 224 peptide bonds per protein molecule (N). By combining Eqs. (4) and (5) and inserting the calculated values for M and N, the following **equation 6** can be obtained:

$$DH\% = 100\left[\left(\frac{\Delta Abs25770d}{6000c}\right) \div 224\right]$$

Therefore:

$$DH\% = \frac{\Delta Abs1.917d}{c}$$

This formula (Eq. 6) was then used for all subsequent calculations of DH by the OPA method.

4.12 Fluorescence Spectroscopy Studies

To evaluate the binding affinity and therefore the possible ability to form complexes between the main milk protein components and polyphenols present in coffee, protein-binding studies were

performed by tryptophan fluorescence quenching experiments. In particular, the possibility to form complexes between each pure milk protein β-LG, β-CN, α-LA, and lactoferrin (Merck Life Science S.r.l., Milano) with each coffee compound such as chlorogenic acid, caffeine and trigonelline (Merck Life Science S.r.l., Milano) added at increasing concentrations to each milk protein solution. Both protein and coffee compound solutions were prepared in Tris-HCl 50mM, pH 6.3 buffer. The analyses were carried out at 25° C using a spectrofluorometer (LS-55, PerkinElmer) equipped with a thermostated cell (HAAKE C25 P thermostat). The fluorescence emission spectra (250–600 nm) were measured using an excitation wavelength λ_{exc}=280 nm, with a 5 nm excitation and emission slit width and averaged from the accumulation of 10 measurements. The dissociation constant (K_d) for each of the binding experiments was determined by fitting the experimental data with the following **equation 7** (GraphPad Prism 7):

$$\frac{F0-F}{F-Fc} = \frac{[Q]^n}{(Kd+[Q])^n} \quad \textbf{Equation 7}$$

where [Q] is the concentration of the added quenching ligand, F is the measured fluorescence, F_0 the starting fluorescence, Fc the fluorescence of the fully complexed protein, Kd the dissociation constant, *n* is the Hill coefficient. Indeed, *n* is an interaction coefficient, such as when *n* =1 the equation describes a complex formation with one site of interaction, while when 1<n<2 a cooperative interaction occurs (Weiss, 1997)

4.13 Other Analytical Procedures

The total protein concentration was determined according to the Bradford method [1976]. A sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the eluted chromatographic peaks after the protein profile analysis on milk by RP-HPLC and to evaluate the products of the *in vitro* digestion procedure. The electrophoresis was carried out as described by Laemmli [1970] under reducing conditions using a 15% acrylamide-bis acrylamide solution and the Mini-Protean III apparatus, gel size 7×8 cm×0.75 mm (Bio-Rad, Hercules, CA). The markers used were Bio-Rad molecular weight standard, low range (phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa). The proteins were visualized on the gel by Coomassie Blue staining (0.1% Coomassie Brilliant Blue R250 in 50% methanol and 10% acetic acid). After destaining the gels were scanned at 600 dpi resolution, and the gel images were analysed using PDQuest software (Version 7.1.1; Bio-Rad, Hercules, CA, USA) according to the protocols provided by the

manufacturer in order to define band-intensity calibration, band detection, and calculation of molecular mass.

The quantitative determination of lactose was carried out through the Lactose/D-Galactose kit (Boehringer Mannheim / R-Biopharm, Darmstadt) following the instruction in the datasheet. Before the lactose determination, milk samples were treated as follows: 2 g of HQ milk (without coffee) or cappuccino samples prepared with HQ milk both treated with the steam injection at increasing temperatures (60-80°C) was placed into a 100 ml volumetric flask and diluted with 20 ml water. Proteins were precipitated by adding 1.0 ml trichloroacetic acid (3 M). After 10 min incubation was neutralized with NaOH (1 M) and up to 100 ml of water was added to the mixture. This final solution was used for the assay.

4.14 Statistical Analysis

Samples comparison was performed using t-test, two-way ANOVA or factorial ANOVA according to the number of factors and levels involved. The level of significance was set at $p < 0.05$. When factors having more than two levels resulted statistically significant the post-hoc Tukey's test was carried out setting a family-wise level of significance of 5%. All the statistical tests were performed using the framework of the General Linear Model (GLM) with the software Minitab 18 (Minitab Inc., State College, PA, USA).

5. RESULTS AND DISCUSSION

5.1 Protein Profile Analysis on Cappuccino and Foam Samples

In the foaming process, caseins and whey proteins play a fundamental role since they absorb at the air-water interfaces forming a film around the air bubbles that stabilizes them. In light of this, it is important to know the behavior of the protein at the interface, in terms of their structure and their stability toward physical and chemical agents such as temperature, pH, ionic strength and pressure.
The role of milk proteins (caseins and whey proteins) on the foaming process and bubble stabilization can be summarized as follow: as soon as the gas is introduced in the milk, a gas-liquid interfacial film is formed that is stabilized by the surface tension between the gas and the liquid (milk) promoting the foam formation. After steam injection, denatured milk proteins are oriented at the air-liquid interface with their hydrophilic and hydrophobic groups, in particular, the polar groups are rearranged towards the aqueous phase and the nonpolar group towards the non-aqueous. The subsequent interactions among unfolded proteins (electrostatic and hydrophobic interactions, hydrogen bonds) give rise to a strong, viscous, and elastic interfacial film that stabilize the air bubbles (Huppertz, 2010, Ho et al., 2021) (see **Figure 10**).

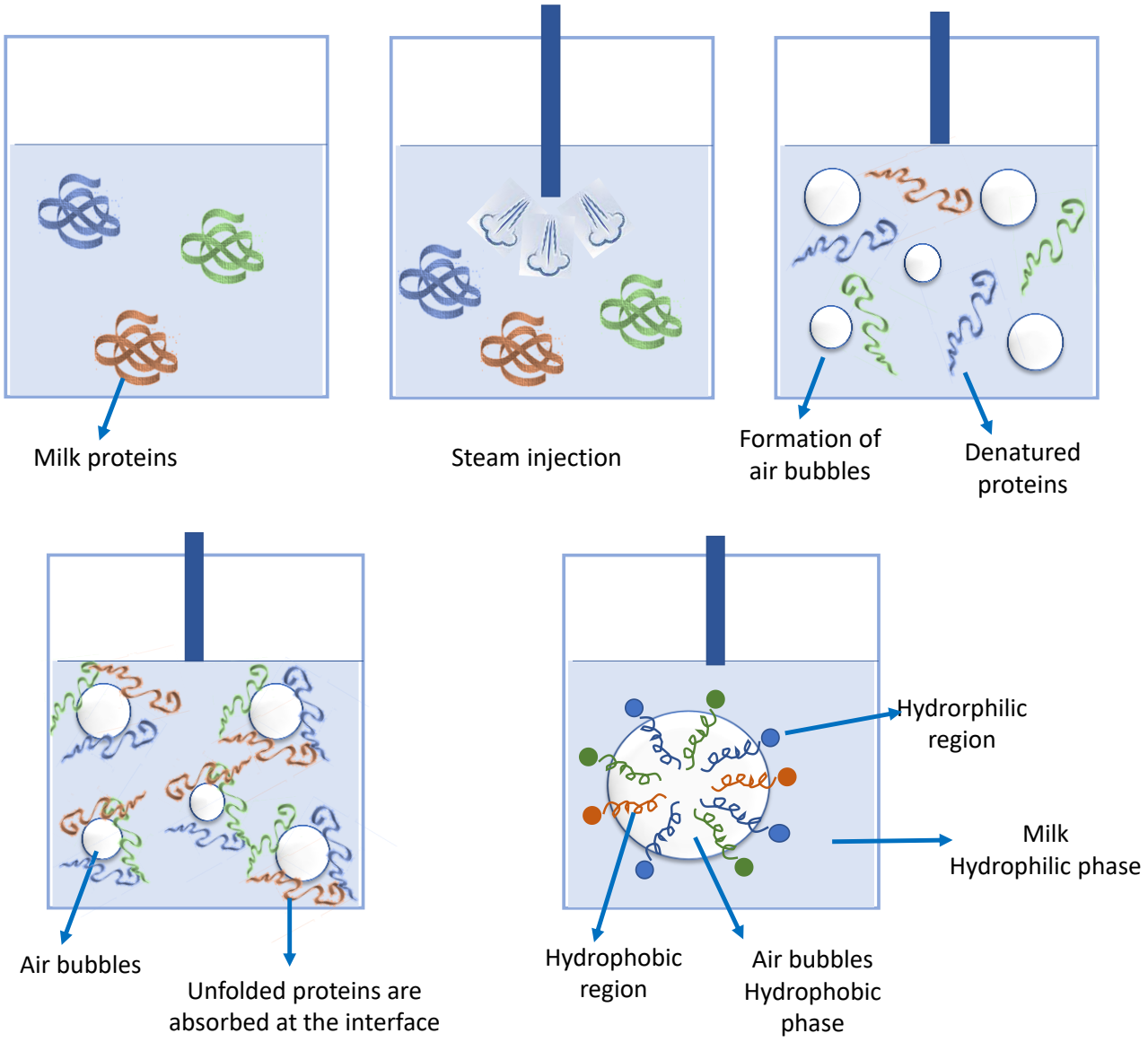


Figure 10. Role of milk proteins in the formation and stabilization of milk-based foams (Adapted from HO et al., 2021).

The behavior of milk proteins at the air-water interface has been the object of several researchers for their importance in some processed foods where it is important to form a consistent foam to enhance its organoleptic properties. These studies took into consideration relationship between the chemical properties of milk proteins with the film formation, surface tension and foam stability (Zang et al., 2004) and evidenced that casein, in particular the unordered β-caseins are characterized by a flexible structure that makes them able to spread and absorb to the interface air-liquid very fast, contrary to the globular whey proteins.

It is well known that the optimum temperature used for the preparation of cappuccino is 60°C. However, in some take away cappuccinos the use of temperatures higher than 60°C for their

preparation is required which, however, can modify the nutritional quality, the organoleptic characteristics, and the foam structure of the beverage itself. It is for this reason that in this work the protein profile of cappuccinos prepared with HQ and UHT milk heated by a coffee machine steamer at different temperatures ranging from 40 to 80°C was studied. In particular, the concentration of β-casein, αs1-casein, β-lactoglobulin, α-lactalbumin and lactoferrin was determined in the following samples: HQ cappuccino, UHT cappuccino, HQ foam, UHT foam. The objective of this study was to deepen the knowledge on the milk proteins behavior in the formation and stability of cappuccino foam.

The protein profile of a commercial pasteurized high-quality (HQ) milk used for cappuccinos preparation, was analyzed by RP-HPLC, and the result is shown in **figure 11**.

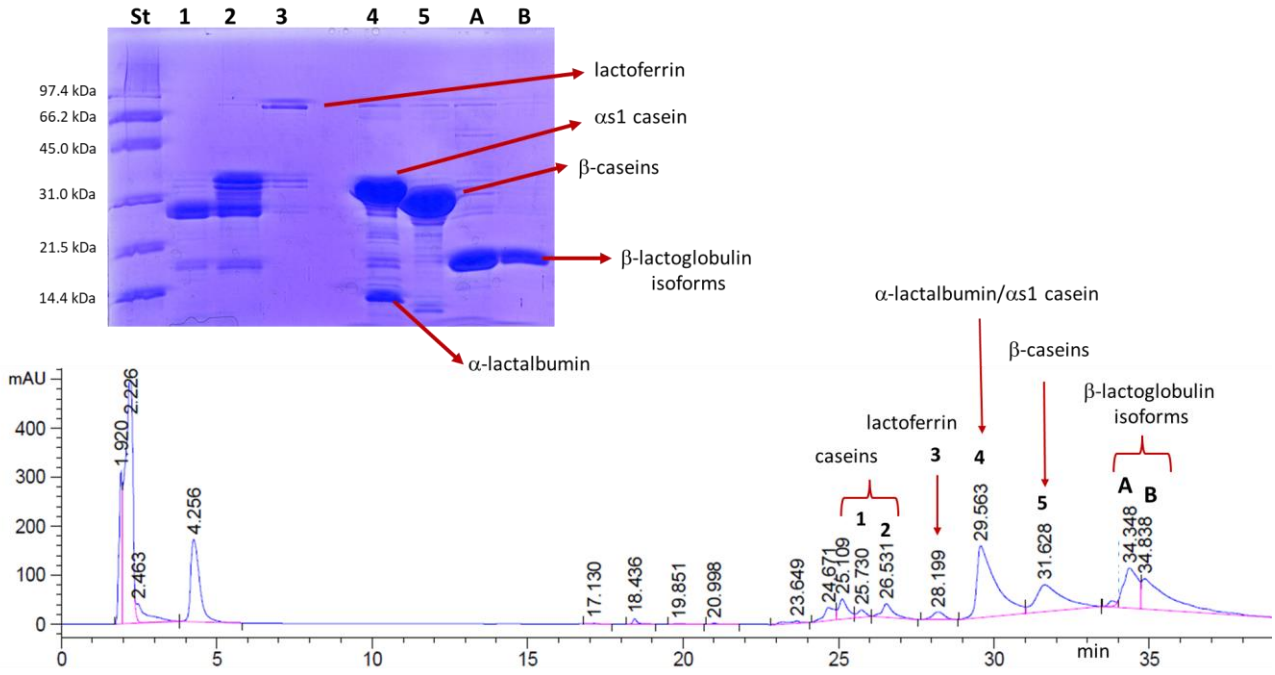


Figure 11. Protein profile of HQ milk after RP-HPLC, inset: 15% SDS-PAGE of the eluted chromatographic peaks

Each peak eluted by the chromatographic runs was analyzed by 15% SDS-PAGE, the electrophoretic gel was subjected to PDQuest analysis to define the molecular weight and the purity percentage of the proteins. Each protein band was extracted from the gel and identified by mass spectrometry analysis. The results showed the presence of caseins (mainly β-caseins and αs1-caseins), β-lactoglobulin, α-lactalbumin and lactoferrin. (**Table 4**).

Table 4. Identification of milk proteins from HQ milk by LC-MS/MS followed by MASCOT software (http://www.matrixscience.com/search_form_select.html) analysis.

Sample ID[a]	Protein name[b]	Score[b]	Mr (kDa)[b]	Mr (kDa)[c]	Sequence
A	β-lactoglobulin (*Bos taurus*)	361	19.8	19.4	VAGTWYSLAMAASDISLLDA
B	β-lactoglobulin (*Bos taurus*)	361	19.8	19.4	VAGTWYSLAMAASDISLLDA
4	αs1-casein (*Bos taurus*)	260	24.5	32.7	HQGLPQEVLNENLLRFFV
4	α-lactalbumin (*Bos taurus*)	61	16.2	13.4	KVGINYWLAHK
5	β-casein (*Bos taurus*)	160	25.1	30.4	PIQAFLLYQEPVLGPVRGPF

Abbreviations:

Mr, molecular mass;

Score number reflects the combined scores of all observed mass spectra that can be matched to amino acid sequences within that protein;

[a]Assigned sample ID as indicated in Figure 2.

[b]MASCOT results (SwissProt databases).

[c]Experimental values were calculated from the electrophoretic gel (15% SDS-PAGE) by the PDQuest software.

Lactoferrin (sample ID 3, as indicated in Figure 2) was identified through the retention time, and the calculation of the molecular weight after SDS-PAGE and PDQuest analysis.

For the quantification of the milk proteins a series of calibration line using standard solution of each β-lactoglobulin, α-lactalbumin, lactoferrin, β-casein and αs1-casein. The equations of these calibration lines along with the respective retention time (RT) and R^2 values are shown in **Table 5**.

Table 5. Retention time (RT) and equation of the calibration lines for each milk protein standard together with the respective R^2 values.

Protein	RT (min)	Equation	R^2
β-lactoglobulin	34.3-34.8	y = 3150.8x – 52.876	0.992
α-lactalbumin	29.56	y = 9859.4x – 479.17	0.981
Lactoferrin	28.20	y = 5086.2x – 32.299	0.986
β-casein	31.63	y = 1164.5x + 126.13	0.943
αs1-casein	29.56	y = 12060x – 3.0452	0.995

The results of the RP-HPLC analyses show that β-lactoglobulin (peaks 1 and 2 of the **Figure 11**) is present as two main isoforms with different retention time (RT): one at RT= 34.3 min and the other at RT=34.62 min. These two isoforms showed similar molecular weight (19.4 kDa) and were judged more than 95% pure (**Figure 11, inset**).

From literature, it is known that β-lactoglobulin exists in two main genetic variants namely A and B and other β-lactoglobulin variants that have been identified by several authors (Gai et al., 2021). The difference between the two variants is the substitutions in variant A of Asp and Val at positions 80 and 134, respectively, by Gly and Ala in variant B (Ng-Kwai-Hang, 1996). Considering this difference between the variant A and B, it can be assumed that the peak with a RT= 34.3 min could correspond to the isoform A which possess the Asp at 80 amino acid position and the peak with a RT=34.62 correspond to the isoform B since it possesses the glycine at 80 position of the amino acid sequence and therefore is more retained by the RP column.

β-casein has been recognized in a peak with RT = 31.7 min and a molecular weight of 30.4 kDa, also in this case this fraction can be considered more that 95% pure. α_{s2}-caseins and α-lactalbumin co-elute in the same peak (RT= 29.6), the respective percentage purity was 71% and 29%, whereas the respective molecular weight was 32.7 and 13.4 kDa. The molecular weight of about 30 kDa observed by SDS-PAGE for the casein fraction is due to their phosphorylation. Lactoferrin has been identified in the chromatographic peak with a RT of 28.3 min, the PDQuest calculated molecular weight was 82.1, consistent with that found in literature (Indyk and Filonzi. 2006) and purity percentage was more than 98%.

In **Figure 12a** is shown the protein profile of a cappuccino prepared with HQ at the standard temperature of 60°C. In addition to the above identified caseins and whey proteins, there are three main peaks, coming from the coffee used for the of cappuccino's preparation, with a RT of 15.9 min,

16.3 min and 23.1min. These peaks were identified by comparing their UV spectra with those of coffee compounds standard solutions. The first one has been identified as chlorogenic acid, the second one as caffeine whereas the third one has not been identified yet (**Figure 12b**).

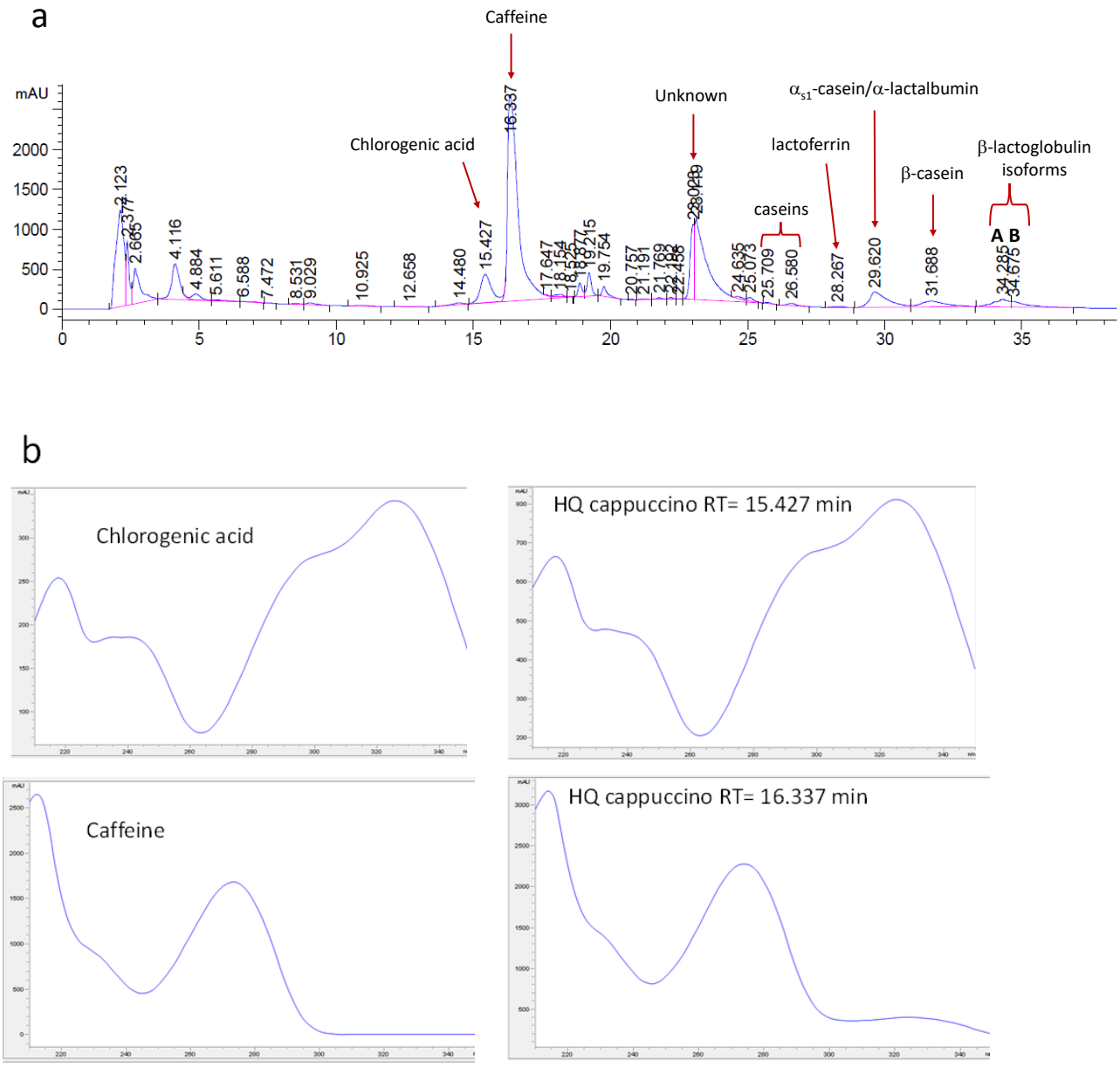


Figure 12. **a**) Protein profile of HQ cappuccino after RP-HPLC. **b**) UV spectra of chlorogenic acid and caffeine and comparison with the chromatographic peaks with RT 15.427 min and 16.337, respectively.

In addition to pasteurized milk (HQ milk) which is the most used for the preparation of cappuccino, the protein profile of cappuccinos prepared with UHT milk was also taken into consideration. In fact, in some cases UHT milk is used for the preparation of cappuccinos because it has a long shelf life

and therefore may result in more practical than pasteurized milk. However, it must be taken in consideration that UHT-milk is characterized by a "cooked" flavor with some sulphureous notes due to protein denaturation induced by the heat treatment.

Several studies evidenced that the foamability of milk systems is enhanced in a milk with high protein content due to the formation of high viscoelastic films at the interface (Thao et al., 2021). Therefore, foams that are prepared with a milk at high protein concentration are denser and more stable.

The total whey protein content of HQ milk is significantly higher than that of UHT milk ($P<0.001$), whereas the total casein content is almost similar in these two types of milk, as shown in **Table 6**. Since whey proteins have an important role in foam formation and stability, the use of UHT milk to produce cappuccino could lead to a final product characterized by poor-quality foam.

Table 6. Protein content in HQ milk and UHT milk

Milk processing	Total whey proteins (mg/ml)	Total caseins (mg/ml)
Pasteurized (HQ)	3.88±0.27 [a]	31.58± 2.90 [a]
UHT treated	0.97±0.26 [b]	32.08±4.10 [a]

Different letters on the same column indicate a statistically significant difference; a, b: $P < 0.001$.

The UHT treatment, in fact, reduces the foam density since can cause a partial denaturation of whey proteins that alters the surface rheological properties reducing the stability of the foam (Thao et al., 2021). Therefore, the foams produced from pasteurized HQ milk are much more stable than those produced from UHT treated milk (Kamath et al. 2008; Sarkar and Singh, 2015).

The protein profile of UHT cappuccino milk, obtained after the RP-HPLC analysis is shown in **Figure 13**. It can be seen that the ultra-high temperature (UHT) treatment leads to a pronounced decrement of the β-lactoglobulin and lactoferrin content.

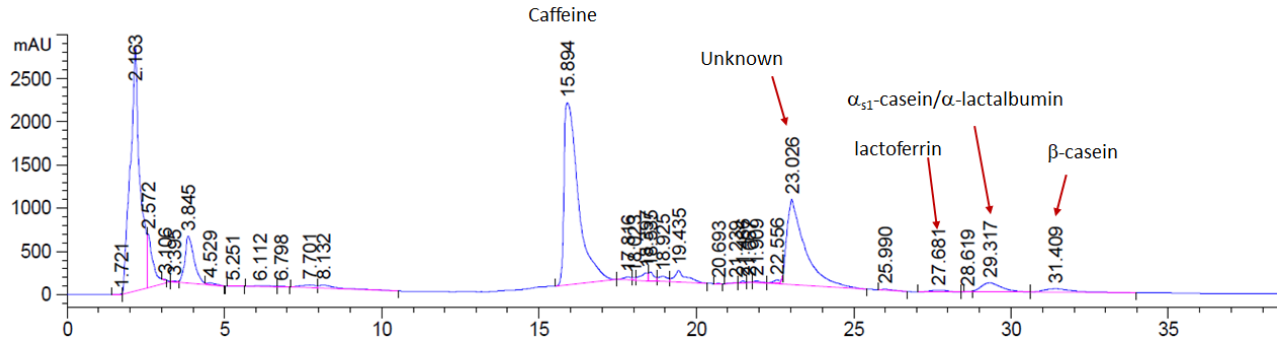


Figure 13. Protein profile of UHT cappuccino after RP-HPLC.

After these preliminary analyses, the concentration of each β-lactoglobulin isoform, β-casein, αs1-casein, α-lactalbumin and lactoferrin was calculated on HQ cappuccino and UHT cappuccino after being heated to different temperatures (from 40 to 80°C) and on the respective obtained foams (HQ cappuccino foam and UHT cappuccino foam).

The results shown in **Figure 14 A** and **B**, indicated that between HQ cappuccino, and HQ cappuccino foam, the concentration of β-casein and αs1-casein is always constant for the whole range of temperatures considered and this occurs also between the UHT cappuccino and UHT cappuccino foam. This indicates that, in general, there are no phenomena of aggregation and precipitation of αs1- and β-caseins induced by temperature and steam injection.

A **B**

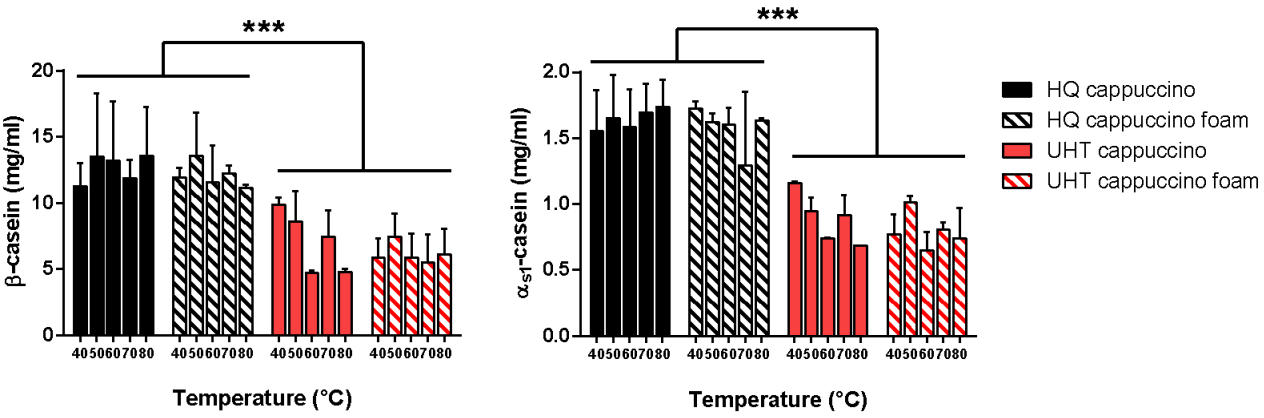


Figure 14. **A**) β-casein and **B**) αs1-casein concentration in HQ cappuccino and HQ cappuccino foam, UHT cappuccino and UHT cappuccino foam at different temperatures obtained varying the steam injection conditions.

However, there is a strong difference in β-casein and αs1-casein between HQ and UHT milk due to the different heat treatment (pasteurization or ultra-high temperature). The difference should be due to the steam injection treatment to which the milk subjected: UHT milk may be more sensitive to the steam injection treatment because it has been subjected to the thermal UHT treatment. This is evident also in the main effects plots shown in **Figure 15 A** (β-casein) and **B** (αs1-casein).

A **B**

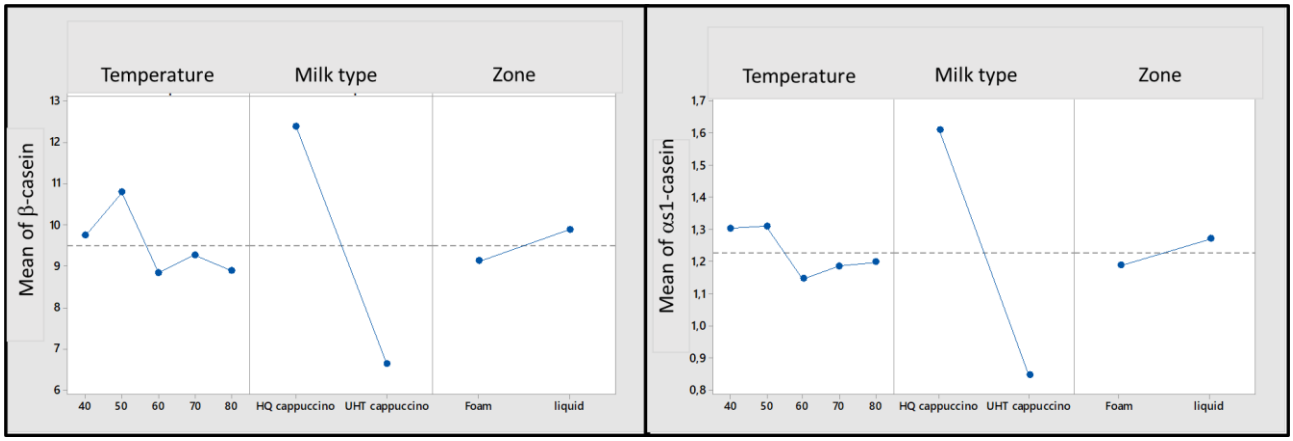


Figure 15. Main effects Plot for **A**) (β-casein and **B**) αs1-casein. Temperature: effect of increasing temperature by steam injection; Milk type: HQ or UHT milk; Zone: liquid or foam phase of cappuccino.

In milk caseins are organized to form macromolecular aggregates named casein micelles (Vincenzetti et al., 2016), however, during cappuccino preparation, the steam injection causes the breakdown of the micelles and the release of the individual caseins. Therefore, in the foam at the interface air-liquid, there is the presence of non-micellar β-casein which can occupy a larger surface than intact casein micelles (Zhang et al., 2004). As discussed before, milk proteins are essential for the stabilization of the interface between air and water in the air bubbles of the milk foam produced by steam injection, the protein in fact are present on the surface of the bubbles where they form a surface layer with the aim to stabilize them since they reduce the surface tension (Xiong et al., 2020). In fact, the milk proteins present at the interface unfold and direct the hydrophilic and hydrophobic groups toward the liquid and the air phase respectively, this forms a film that stabilizes the bubble. In a study carried out by Ipsen and Otte (2004) the relationship between the structure of purified milk proteins and their ability to form a stable film on the air-water interface of the foam has been investigated. Caseins are less structured and more flexible proteins than the globular whey proteins and therefore, they can occupy a larger area at the interface which results to be soft and compressible.

Among caseins, β-casein has a less ordered secondary structure with respect to α_{s1}-casein (**Figure 16**), therefore diffuses more extensively at the interface thanks to the hydrophobic amino acid region anchored at the layer surface and the hydrophilic region directed towards the aqueous phase. α_{s1}-casein, which shows random distribution of the hydrophobic and hydrophilic residues, has a smaller distribution at the interface with respect to β-casein (Ipsen and Otte, 2004). α_{s1}-casein, more structured with respect to β-casein, forms more voluminous and stable foam with respect to β-casein but is not able to form a strong interfacial layer (Ipsen and Otte, 2004).

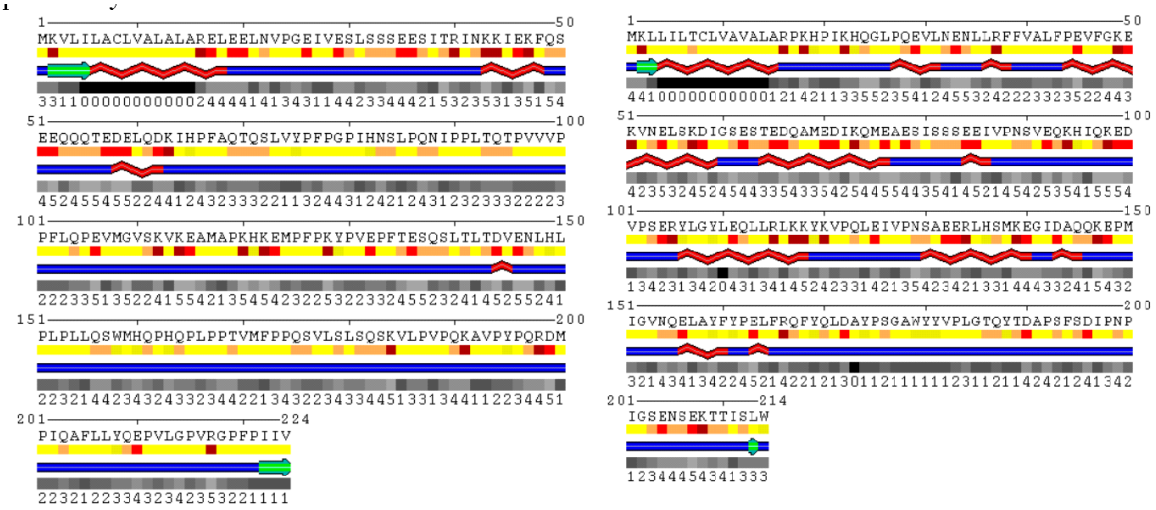


Figure 16. Secondary structure prediction of β- and α_{s1}-casein. α-helices; beta strand; random coil; 0123456789 relative solvent accessibility, where 0 means completely buried and 9 fully exposed; HAPNC H-hydrophobic, A-amphipathic, P-polar, N/C-charged. The secondary structure prediction was performed at http://minnou.cchmc.org/.

Unlike caseins, whey proteins have a globular structure and are characterized by the presence of secondary, tertiary and, in some cases, quaternary structure. β-lactoglobulin, α-lactalbumin, bovine serum albumin, lactoferrin, immunoglobulins and other minor proteins are part of the whey proteins. The difference between the soft, unstructured caseins and more structured whey proteins are responsible for the foaming properties of milk.

Martin and co-workers (2002) have demonstrated that at a protein concentration of 0.1-3.0% (w/v) and pH 6.7, β-lactoglobulin showed a lower foamability with respect to β-casein, because of its self-association that produce a dimer with a higher molecular size and its structural rigidity. However, β-

lactoglobulin produced more stable foam than β-casein because of the strong internal structure, low adsorption probability and the presence of free disulfide groups which form a rigid network at the interface.

As discussed before, β-lactoglobulin is known to exist in two main genetic variants namely A and B, the difference is the substitutions in variant A of Asp and Val at positions 80 and 134, respectively, by Gly and Ala in variant B. This can give rise to a different hydrophobicity of the two variants responsible for the different retention time of the RP-HPLC.

Considering the β-lactoglobulin concentration behavior in HQ cappuccino (liquid phase), the results indicated that the **isoform A** and **B** seems not to be greatly affected by the heat treatment carried out by the steamer throughout the temperature range considered (**Figure 17 a** and **b**). However, the concentration of isoforms B were significantly lower ($P<0.0001$) in the HQ cappuccino foam (foam phase) with respect to the HQ cappuccino (liquid phase) as also shown in the main effects plot for β-lactoglobulin A and B. (**Figure 18 a** and **b**).

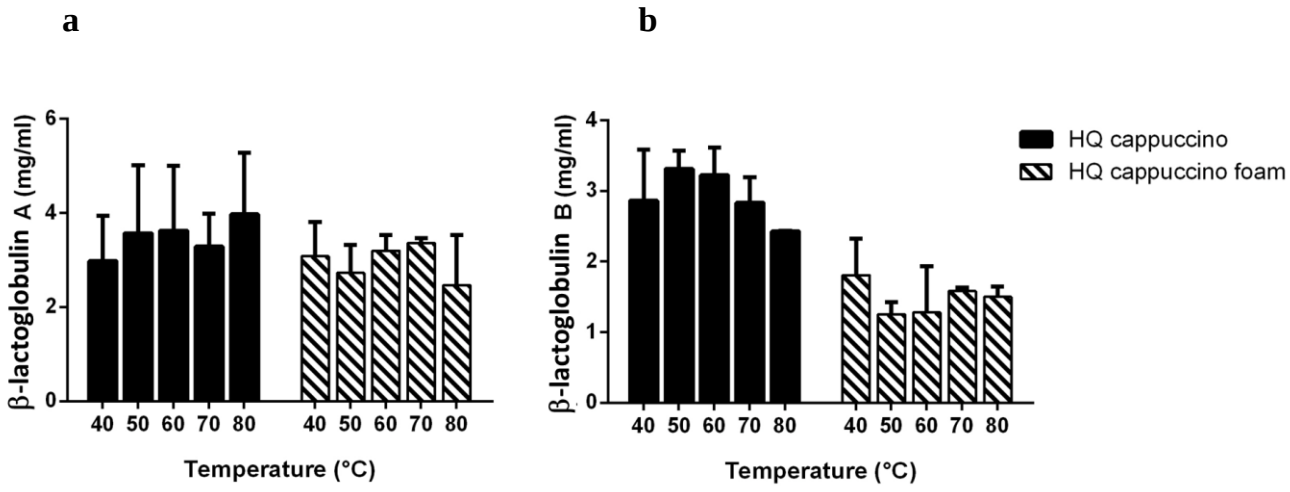


Figure 17. β-lactoglobulin isoform A (**a**) and isoform B (**b**) concentration in HQ cappuccino and HQ cappuccino foam at different temperature and steam injection conditions.

a **b**

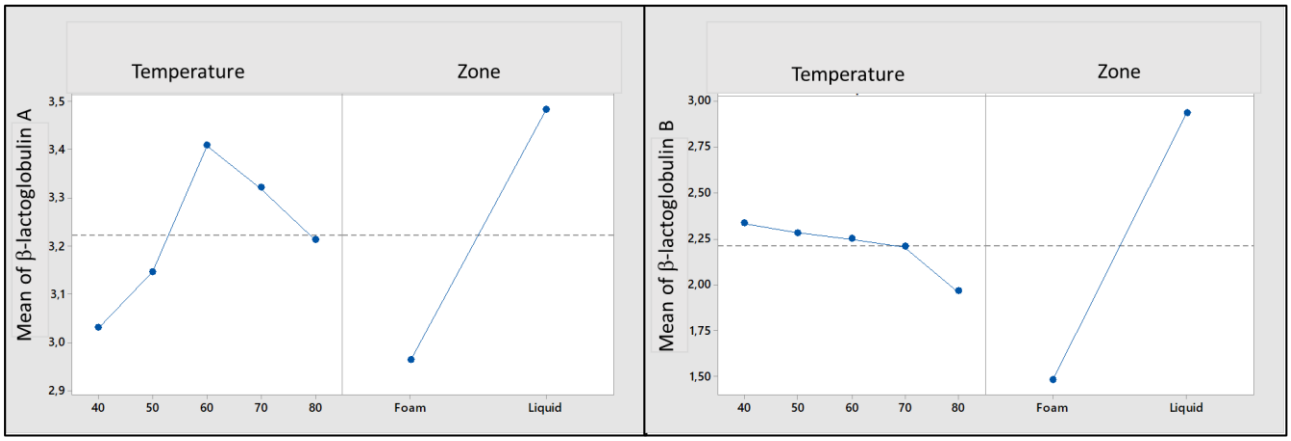


Figure 18. Main effects Plot for **a**) β-lactoglobulin A and **b**) β-lactoglobulin B. Temperature: effect of increasing temperature by steam injection; Zone: liquid or foam phase of cappuccino.

Both variants A and B are responsible for the foam stability thanks to their ability to form intermolecular disulfide bonds at the interface. Isoform B rapidly forms a stronger interfacial layer giving rise to a more stable foam than the A variant (Ipsen and Otte, 2004).

The observed decreased concentration of the β-lactoglobulin isoform B, may be due to the formation of unfolded protein that allows hydrophobic interactions and the compaction of the protein molecules at the interface. This could result in a strong interfacial film and good bubble coverage. The greater foam stability can be due to an increase in the viscosity of the protein solution because of the presence of aggregates that slowed the rate of liquid drainage due to the interfacial film compacting (Sarkar and Singh, 2015; Ho et al., 2021).

β-lactoglobulin was not observed in the UHT cappuccino and UHT cappuccino foam samples, indicating that the ultra-heat treatment negatively affected the content of this protein.

The fact that industrially applied processing methods, such as homogenization, fat separation, pasteurization, and ultra-heat treatment led to a decreased amount of some milk protein has been well described by several authors. β-lactoglobulin, as well as other whey proteins, is affected by the heat treatment that occurs during UHT processing (Krishna et al., 2021).

In this work it has been found that α-lactalbumin concentration is not affected by the increasing temperature after the steam injection treatment. This is consistent with the literature where it is reported that at the temperature of 63.7°C (which is close to the optimal temperature for a cappuccino preparation), α-lactalbumin is denatured but doesn't aggregate. However, α-lactalbumin concentration is slightly lower ($P<0.005$) in the foam phase samples with respect to the respective

liquid phase samples, and this occurs in both HQ and UHT milk, as shown in the main effects plot (**Figure 19**).

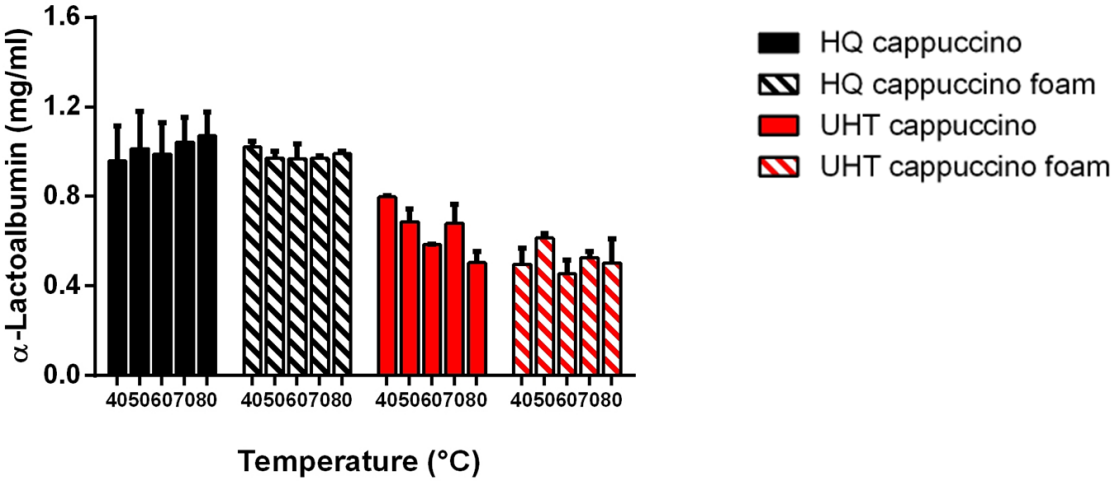


Figure 19. α-lactalbumin concentration in HQ cappuccino and HQ cappuccino foam, UHT cappuccino and UHT cappuccino foam at different temperatures obtained varying the steam injection conditions.

The main effects plot (**Figure 20**) shows also that the α-lactalbumin concentration is significantly lower ($P<0.0005$) in the HQ milk with respect to the UHT milk and also in this case, this is due to the different heat treatment.

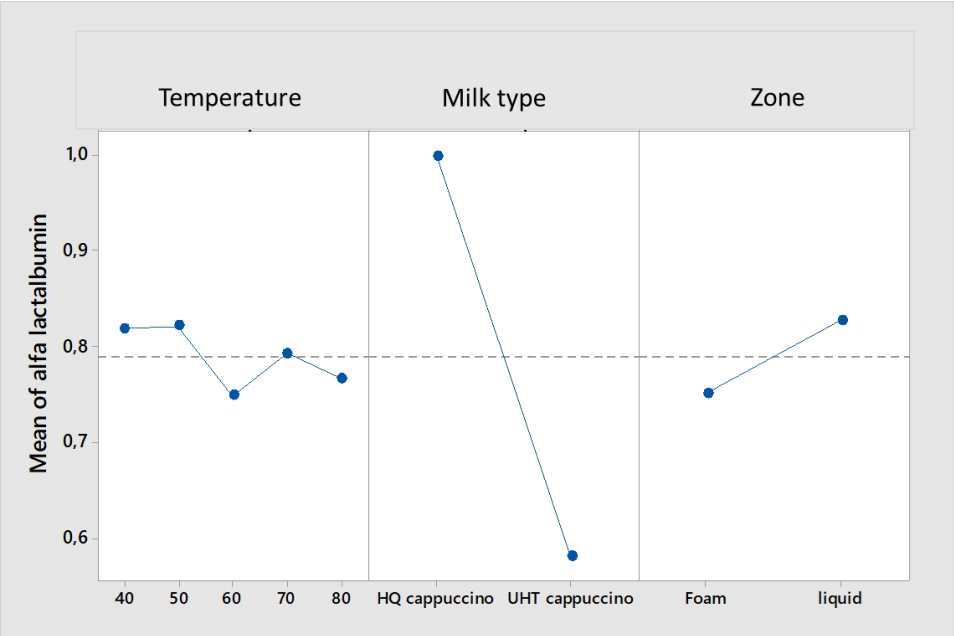


Figure 20. Main effects Plot for α-lactalbumin. Temperature: effect of increasing temperature by steam injection; Milk type: HQ milk or UHT milk; Zone: liquid or foam phase of cappuccino.

At the temperature and steam injection conditions used to prepare the cappuccino α-lactalbumin may denature but not aggregate, therefore this protein can absorb at the air-liquid interface but, differently from β-lactoglobulin, is not able to form a strong interfacial film. This agrees with the observations of Ipsen and Otte, (2004) who have proved that α-lactalbumin, more structured than β-lactoglobulin, is able to form a voluminous but unstable foam.

Lactoferrin concentration is significantly affected by the milk type, since it is lower in the UHT milk with respect to HQ milk ($P<0.0005$) as shown in **Figure 8** and in the main plot effects **(Figure 21**). Furthermore, in the foam samples (both in the cappuccinos prepared with UHT milk and those prepared with HQ milk) the concentration of this protein is significantly lower than that found in the liquid phase ($P<0.0005$).

The concentration of lactoferrin in the HQ cappuccino foam sample is also affected by the increased temperature by steam injection, especially at 80°C ($P<0.0005$) as shown by the main effects plot (**Figure 22**).

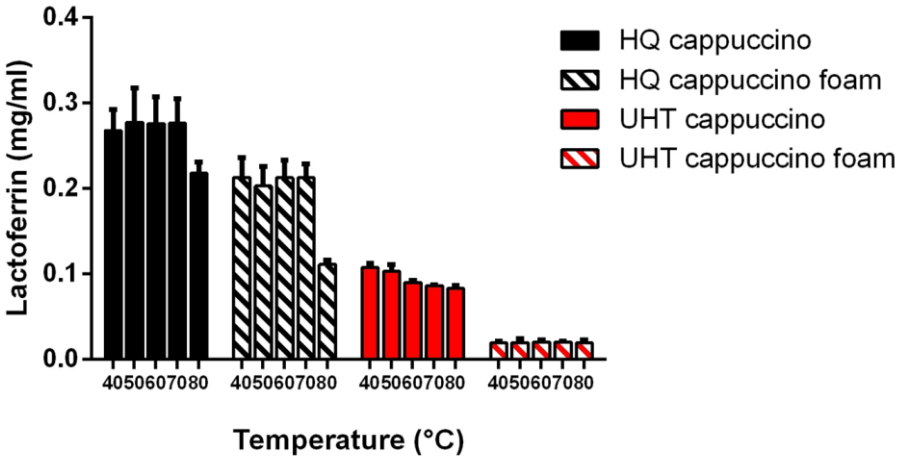


Figure 21. Lactoferrin concentration in HQ milk, HQ cappuccino and HQ cappuccino foam at different temperature and steam injection conditions.

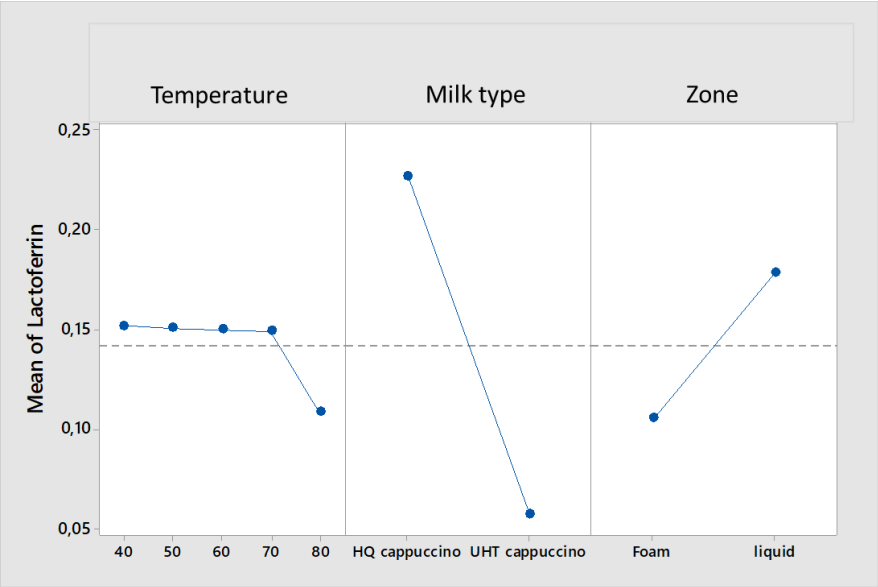


Figure 22. Main effects Plot for Lactoferrin. Temperature: effect of increasing temperature by steam injection; Milk type: HQ milk or UHT milk; Zone: liquid or foam phase of cappuccino.

Lactoferrin is a glycoprotein of about 80 kDa which is present at low levels in cow's milk but has an important iron-binding ability. This protein belongs to the transferrin protein family which also includes serum transferrin, ovotransferrin, melanotransferrin and the inhibitor of carbonic anhydrase (Giansanti et al., 2016). Lactoferrin is involved in several physiological and protective functions,

since it shows antimicrobial, anti-inflammatory, anticancer activity and regulates the iron absorption in the bowel. Its antimicrobial activity is due to its ability to remove iron from potential pathogens.
From literature it is known that lactoferrin exhibits good foam formation but poor foam stability properties, (Dai et al., 2021). Other studies showed that lactoferrin can form complexes with some polyphenols such as proanthocyanidins, epigallocatechin gallate, and chlorogenic acid and some of them led to a better foamability and foam stability than the lactoferrin alone (Zhang et al., 2019; Li et al., 2020). In another work the complex between lactoferrin and tannic acid has been studied and it was shown that the complexes were more hydrophilic and larger than the lactoferrin alone and this made them absorb more slowly to the air–water interface leading to a reduced foamability. However, the lactoferrin-acid tannic complexes are able to form a thick viscoelastic layer around the bubble that increases the foam stability (Dai et al., 2021).
In this work, it was found that the lactoferrin concentration decreased in the cappuccino foam and this could be due to the possible formation of aggregated or denatured protein or to the formation of complexes with compounds derived from the coffee that may be involved in the foam formation and stability.
It is important to take into account, when interpreting these results, that the decreased concentration of β-lactoglobulin and lactoferrin observed in the cappuccino foam samples is not due to the different system (air bubble) that occurs in the foam phase with respect to the liquid phase of cappuccino since the concentration other milk proteins such α_{s1}-casein, and β-casein is the same in both phases (see **Figures 18** and **22**) An explanation of the decreased concentration of β-lactoglobulin and lactoferrin in the foam phase observed in this study, could be due to protein aggregation at the air-milk interface in the cappuccino foam. When foam samples are subjected to RP-HPLC, these aggregates of protein can precipitate after the centrifugation to which samples are subjected before the chromatographic run. In light of this, it could be supposed that the decreased concentration of β-lactoglobulin and lactoferrin that has been found in the cappuccino foams through these experiments, could be considered as indirect evidence that the aggregation process of denatured proteins at the air-liquid interface has taken place.

5.2 Lactoperoxidase Activity

LPO is a member of the peroxidase-cyclooxygenase superfamily and one of the most abundant enzymes in bovine milk, making up about 1% of the whey proteins in milk. LPO catalyzes the oxidation of various inorganic and organic substrates by hydrogen peroxide. This enzyme, together with its substrates, hydrogen peroxide and oxidized products forms the "lactoperoxidase system". Oxidized products produced through the action of this enzyme exhibit bactericidal and antiviral activities. LPO is therefore an antioxidant enzyme and is a natural antimicrobial system (Sharma et al., 2013). It can be found in the secretions of mammary glands, salivary and lacrimal glands and has the function to protect the glands and the milk itself against pathogens, and the intestinal tract of infants against pathogenic microorganisms which may be present in milk. In general, the presence of active LPO ensures a longer shelf-life of the milk.

Figure 23 shows the behaviour of lactoperoxidase (LPO) activity, expressed as mU/mg of protein, of HQ milk sample treated with the steam injection in the temperatures range of 50-80°C. It is well known from the literature that the pasteurization process does not inactivate LPO contrarily to the UHT treatment (Ostdal et al., 2000, Silva et al., 2020). In the unheated HQ milk, LPO activity was 7.96±0.6 mU/mg.

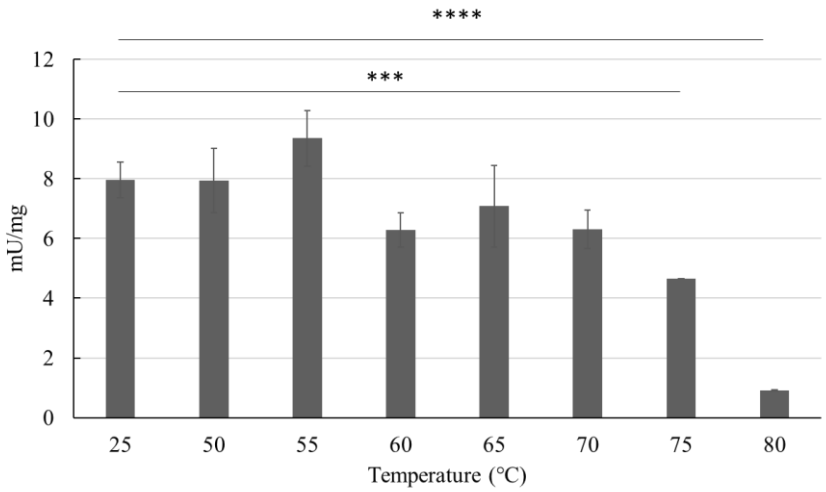


Figure 23. Lactoperoxidase activity of HQ milk sample at 25°C and subjected to steam injection treatment in the temperature range of 50-80°C. *** P<0.005; **** P<0.001.

The fact that at the cappuccino preparation temperature (60-65°C) LPO is still active is very important to ensure a good quality of the beverage itself.

5.3 Water-Soluble Vitamins Determination on Pasteurized Milk Subjected to Heating and Steam Injection Treatment

Vitamins are organic compounds required by the body in small amounts. The best way to get vitamins necessary for body functions is to eat a balanced diet with a variety of foods. Vitamins are required for metabolism, protection, maintenance of health, and proper growth. Some of them are part of coenzymes which are essential for the correct execution of several enzymatic reactions that occur in metabolism. These micronutrients can be divided into water-soluble and fat-soluble vitamins. Among water-soluble vitamins are the B vitamins (B1, B2, B3, B5, B6, B8, B9, B12) and vitamin C, they are not stored in the body and are excreted by the kidney and require daily food intake. This class of vitamins can be found in several vegetable and animal foods, milk, and its derivatives (Brancaccio et al., 2022). The deficiency of vitamins can be primary if the diet does not ensure a correct intake of vitamins or secondary if the cause is a condition (for instance a disease) that limits or prevents the absorption of vitamins from food. In both cases, hypovitaminosis can lead to severe pathological conditions. Very recently, some authors found that vitamins have an important role in COVID-19 and immunodeficiencies because they act by activating the immune system and stimulating lymphocytes (Jovic et al., 2020; Scudiero et al., 2021). Concerning the vitamin B content in cow's milk, vitamin B1(Thiamine), vitamin B3 (niacin), and vitamin B6 ranged from 0.59–2.37 μM, 2.43–16.24 μM, and 1.0–11.23 μM, respectively (INRAN, 2006; Graulet and Girard, 2017; Schmidt et al., 2017). Cow's milk vitamin B2 (Riboflavin) content ranges from 2.12 to 6.91 μM and showed a seasonal variation content with the highest value during the wintertime. In pasteurized whole bovine milk (HQ milk), the vitamin content is around 4.8 μM [INRAN, 2006; Poulsen et al., 2015]. The content of vitamin B9 (folic acid) in bovine milk is highest in the colostrum (0.99 μM), but during the lactation period slowly decreases reaching the concentration of 0.18 μM at 39 days of lactation. It should also be taken into consideration that the quantity of folic acid in cow's milk is influenced by several factors, for instance, cattle nutrition, the presence of antioxidant vitamins, and the presence of oxygen (INRAN, 2002). Considering the vitamin B12 content in cow's milk, some authors found a correlation with the animal genetic variants: the cow genotype influences the microbial population composition of the rumen microflora which is responsible for the synthesis of most vitamin B12. Therefore, the concentration of vitamin B12 shows great variability, for example in Dutch Holstein Friesian cow's milk varied from 0.73 x 10^{-3} μM to 93x10^{-3} μM (Rutten et al., 2008).

From literature, it is known that milk pasteurization causes a loss of some vitamins sensitive to the heat treatment, mainly ascorbic acid (18,7%), folates (12%), vitamin B1 (3%), and also vitamin B12 (10%). No change in vitamin B2 (riboflavin) after pasteurization treatment was observed (Andersson, and Öste, 1994)

The vitamin content determination was carried out in pasteurized HQ milk normally used to prepare cappuccinos, the milk samples were thermally treated at increasing temperatures or were subjected to steam injection for different seconds (from 20 to 60 seconds). In this way, it was possible to establish if any changes in vitamin content were due to the increasing temperature only, to the steam injection, or both. UHT milk was not used in these experiments because it is supposed that heat treatment to which the milk is subjected leads to a drastic decrease in the vitamin content.

Linearity was assessed by building external calibration curves for each water-soluble vitamin using vitamin standard solutions. **Table 7** shows the retention times, and the equation of the calibration lines for each water-soluble vitamin standard, with the respective correlation coefficient (r^2) values. Correlation coefficients ($r2$) > 0.99 were obtained for all compounds studied. The vitamins in the milk samples were identified by comparison of their retention times.

Table 7. Retention time (RT) and equation of the calibration lines for each water-soluble vitamin standard together with the respective r^2 values.

Standard water-soluble vitamins	RT (min)	Equation	r^2
Thiamine (vitamin B1)	24.2-24.6	y=33.53x-3.8732	0.992
Riboflavin (vitamin B2)	19.4-19.9	y=121.82x-0.9437	0.999
Nicotinic acid	4.27	y=15.323-0.3843	0.999
Nicotinamide	5.8-6.0	y=10.343x+3.4316	0.995
Pyridoxine (vitamin B6)	8.14	y=11.605x-3.3578	0.999
Folic acid	12.2-13.4	y=110.71x-15.488	0.999

The vitamins determined in the HQ milk samples before thermal treatments were nicotinic acid (11.05±4.1 µM), nicotinamide (10.14±0.68 µM), vitamin B6 (1.58±0.38µM), vitamin B2 (3.40±0.14), and folic acid (0.45±0.1 µM).

When the HQ milk was subjected to the heat treatment (no steam injection), it was found that the concentration of the five water-soluble vitamins, was not affected (**Figure 24**).

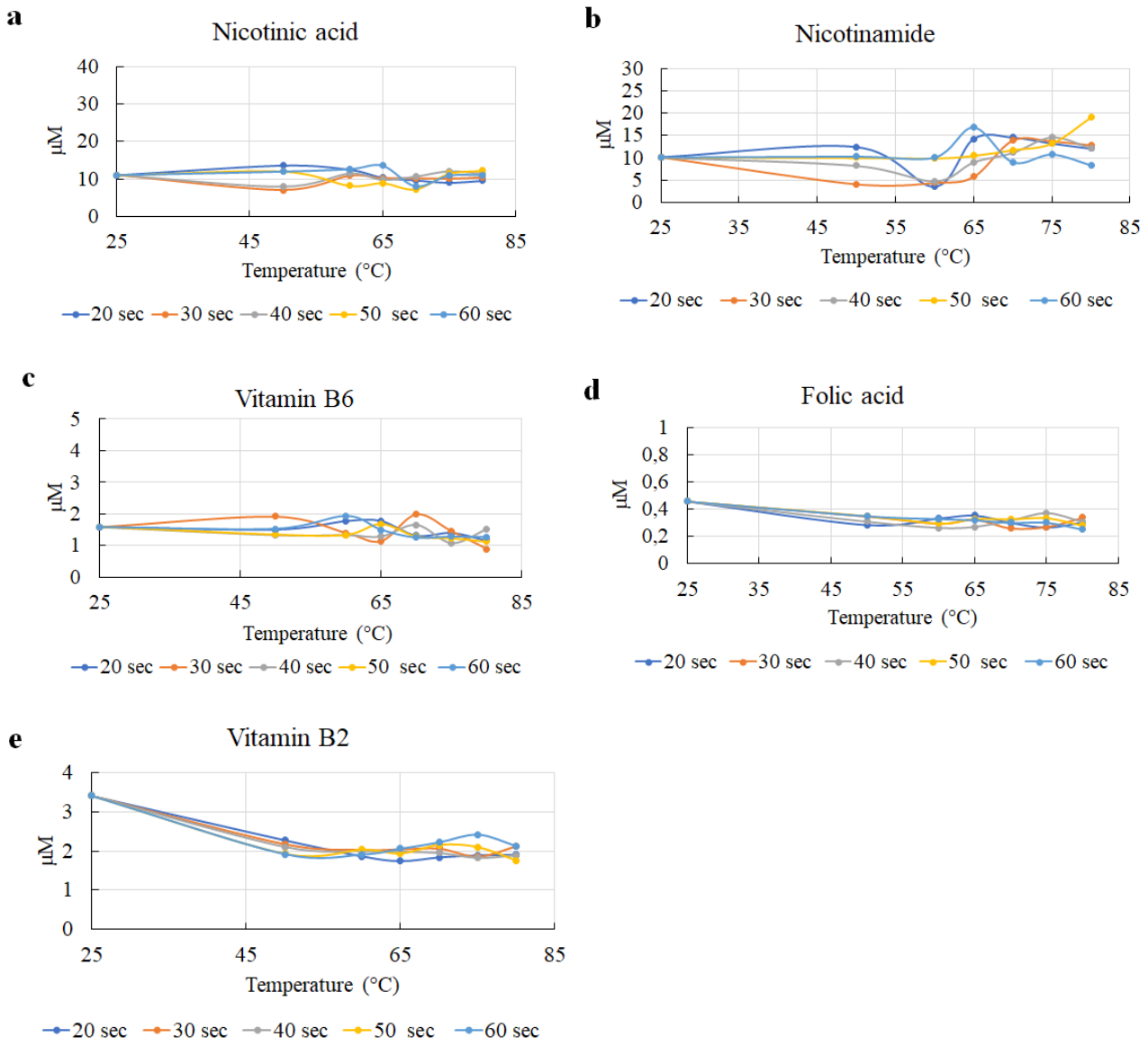


Figure 24. Concentration of **a**) Nicotinic acid, **b**) Nicotinamide, **c**) Vitamin B6, **d**) Folic acid, and **e**) Vitamin B2 in HQ milk subjected to heat treatment (from 50 to 80°C for 20, 30, 40, 50, 60 seconds).

However, when the HQ-milk is treated with steam injection at increasing temperatures, an interesting result regarding the nicotinic acid concentration was obtained. In fact, the concentration of nicotinic acid increases by about 5 folds starting from 11.05 µM at 25°C to reach a mean value of about 40-50 µM at a temperature range of 60-70°C ($P<0.005$). It is also interesting to note that the increment of nicotinic acid concentration is time-dependent: if the HQ-milk is treated for 20 seconds with the steam injection (time normally used to heat the milk during the preparation of the cappuccino), nicotinic acid reaches its highest concentration (58 µM) at 70°C, but if the milk is treated for 60 seconds, the

highest concentration (45 µM) is reached at 50°C (**Figure 25**). As the concentration of nicotinic acid increases, so the concentration of nicotinamide decreases. This form of vitamin B3 already decreases at a temperature of 50°C after 30 seconds of treatment with the steam injection and it is practically absent at higher temperatures.

The same behavior was observed for the folic acid concentration. However, during the preparation of the cappuccino, the temperature of 75°C is never reached. Finally, vitamin B2 was not affected by the steam injection treatment. All these results indicated that the concentration of water-soluble vitamins, seems not influenced by the steam injection at the temperature normally used for the cappuccino preparation (about 60°C).

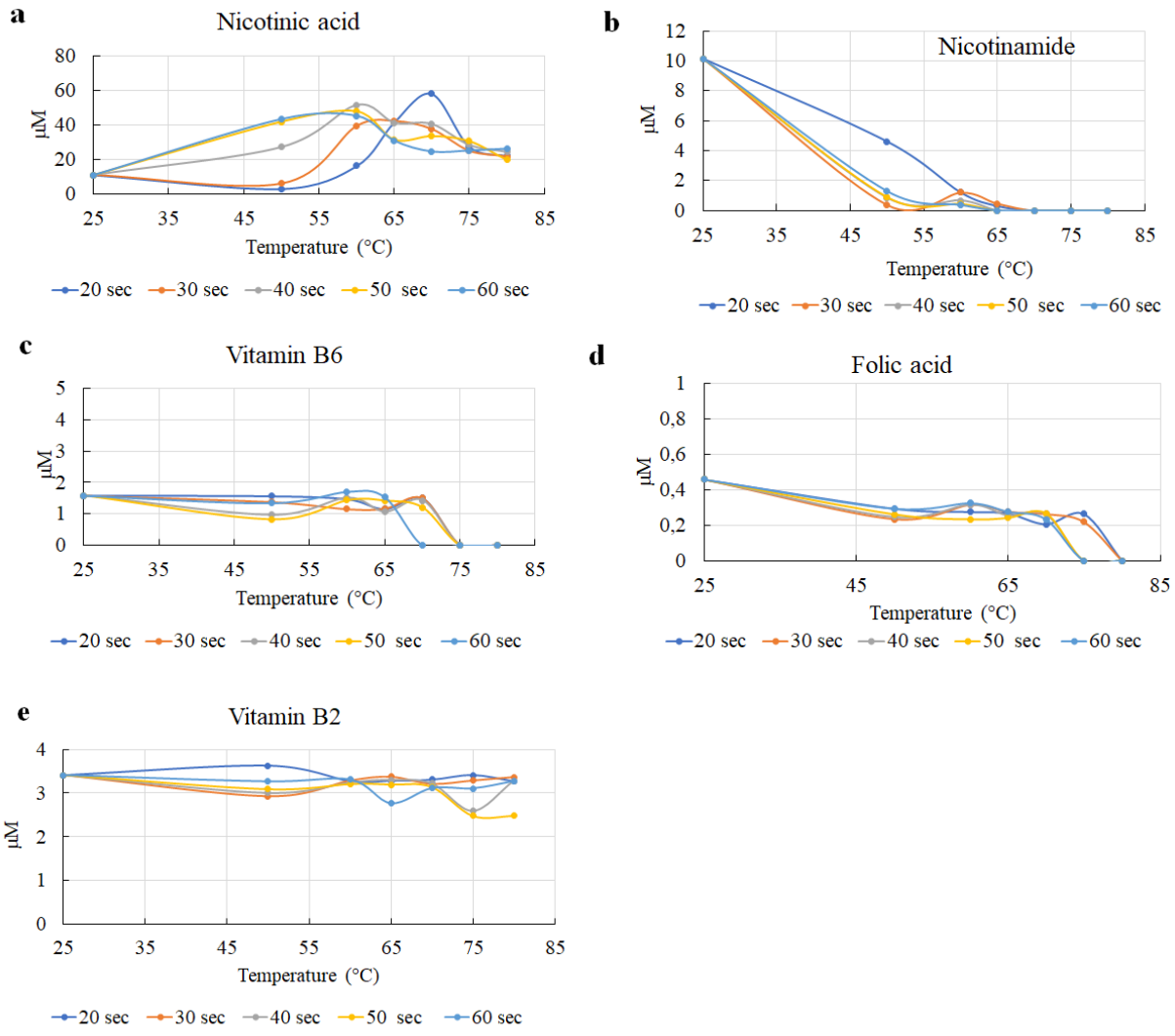


Figure 25. **a**) Nicotinic acid, **b**) Nicotinamide, **c**) Vitamin B6, **d**) Folic acid, and **e**) Vitamin B2 content in HQ milk subjected to steam injection (from 50 to 80°C for 20, 30, 40, 50, 60 seconds).

The increase of nicotinic acid concentration in milk samples after steam injection is accompanied by the simultaneous decrease of nicotinamide (**figure 25 b**) however, the process that leads to the formation of nicotinic acid after steam injection is not yet clear and this aspect should certainly be investigated. The increment of nicotinic acid content is very interesting considering the beneficial effects on human health exerted by this vitamin.

Nicotinic acid (Niacin) is used to prevent and treat niacin deficiency that may result from alcohol abuse, malabsorption syndrome, Hartnup disease or poor diet. Furthermore, nicotinic acid is used to treat dyslipidaemias since it reduces triglycerides and LDL cholesterol and raises HDL cholesterol levels (Bogan and Brenner, 2008). Furthermore, niacin seems to be involved in the modulation of inflammation with a beneficial effect on adipokine expression and inflammatory markers. The biologically active forms of niacin are nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) which act as coenzymes of many oxidoreductases, intervening in most of the electron and H^+ transfer reactions in the metabolism of carbohydrates, acids fats, and amino acids. Folic acid is an important B vitamin involved in purine and pyrimidine biosynthesis (DNA and RNA) and amino acid interconversions and it is known that this vitamin exerts a protective role against neural tube defects occurring during early pregnancy (Daly et al., 1995). Furthermore, other authors suggested that there is an association between folate intake and a reduced risk of cardiovascular diseases. It has been shown that a low folate concentration is correlated with elevated homocysteine plasma concentration, which is in turn, correlated with elevated risk for coronary heart disease (Boushey et al., 1996). Other evidence showed that low folate concentration increases the risk of colon cancer (Mason, 1995).

Vitamin B2 (riboflavin) has a mainly coenzymatic function and, in its metabolically active forms (FMN and FAD), constitutes the prosthetic group of redox enzymes called flavoenzymes or flavoproteins necessary for the metabolic pathways of carbohydrates, lipids, and amino acids.

5.4 Fat-Soluble Vitamins Determination on Pasteurized Milk Subjected to Heating and Steam Injection Treatment

Contrary to water-soluble vitamins, the transport and absorption of fat-soluble vitamins is closely associated with that of lipids. Like lipids, this class of vitamins requires the presence of bile salts for absorption and their transport occurs through the chylomicrons. Furthermore, fat-soluble vitamins can be stored with the fats.

In milk, the fat-soluble vitamins present in greater quantities are vitamins A, E and K. The content of these vitamins is influenced by the food ration composition of the dairy cow and also by the season and the lipid content (Mandrioli et al., 2020). In bovine milk the content of vitamin A is in the range of 0.35-3.14 μM (INRAN, 2006; Haug et al., 2007), this vitamin is found in milk and derivatives mainly in the form of retinyl ester and its content varies according to the diet, the lactation period, the climatic conditions, and breeding. The average intake of vitamin A from milk is about 57 mg/day which corresponds to 8% of the daily requirement (INRAN, 2006). Following the UHT treatment of the milk, cis isomers of the retinol esters are formed which are due precisely to the heat treatment and a decreased amount of vitamin A has been observed. On the contrary, the concentration of vitamin A in pasteurized milk (HQ milk) remains stable with the same concentration found in raw milk, as observed by Maguer and Jackson (1983). In bovine milk the most present form of vitamin E is α-tocopherol. The average content of α-tocopherol in milk is in the range of 0.46 to 1.62 μM. The greatest quantity of vitamin is present in the colostrum, which concentration is approximately 4.41 μM, but it rapidly decreases within a few days until it reaches the value of raw milk (INRAN, 2006). Like all fat-soluble vitamins, the concentration of vitamin E is also influenced by the amount of fat present in milk and is also affected by the conditions of livestock farming. Regarding the stability of this vitamin following heat treatments of milk, it has been shown by Niero and co-workers (2018) that vitamin E, in this study calculated as the sum of α-tocopherol and γ-tocopherol, was similar in whole pasteurized and raw milk, as a mean concentration of averaging 3.64 and 3.62 μM, respectively, whereas in the whole UHT was 3.08 μM. The concentration of vitamin K in bovine milk is ranging from 5.66 nM to 15.3 nM. High concentrations of vitamin K are found in colostrum, but then decrease to the average values of mature milk in a few days. Vitamin D concentration in bovine milk ranges between 0.26 nM and 0.73 μM. Some authors determined the content of vitamin D3 in pasteurized milk (HQ milk) and found that its content varied from 2.6 nM to 0.044 μM (Mandrioli et al., 2020).

In the present work, the vitamin content determination was carried out on pasteurized HQ milk normally used to prepare cappuccino, the milk samples were thermal treated at increasing temperatures or where subjected to stem injection for different seconds (from 20 to 60 seconds). A calibration curve for each fat-soluble vitamin standard was performed and in **Table 8** are shown retention times, the equation of the calibration lines, and the respective correlation coefficient (r^2) values, obtained after the RP-HPLC analysis.

The vitamins in the milk samples were identified by comparison of their retention times.

Table 8. Retention time (RT) and equation of the calibration lines for each fat-soluble vitamin standard together with the respective r^2 values.

Standard fat-soluble vitamins	RT (min)	Equation	r^2
Vitamin A	4.20-4.30	y = 1812x + 28.015	0.98
Vitamin D_3	7.60-8.10	y = 1756.2x – 39.996	0.99
Vitamin E (α-tocopherol)	8.40-9.10	y = 229.45x – 8.0551	0.99
Vitamin K_1	13.4-13.6	y = 1330.7x – 140.23	0.99

The fat-soluble vitamins found in the HQ milk samples, before heating treatment, were as follows: vitamin A, 1.07±0.23 µM; vitamin E, 1.24±0.44 µM; and vitamin D, 0.1 ±0.009 µM. These values agreed with those already reported in literature (INRAN, 2006; Haug et al., 2007; Mandrioli et al., 2020). The concentration of the three fat-soluble vitamins A, and D determined in these experiments, did not change even after the heat treatments to which the HQ milk was subjected, either by increasing the temperature only (**Figure 26**) or through a steam injection for 20-60 seconds, as shown in **Figure 27**. Vitamin E showed a slight decrease both with increasing temperature and with steam injection treatment.

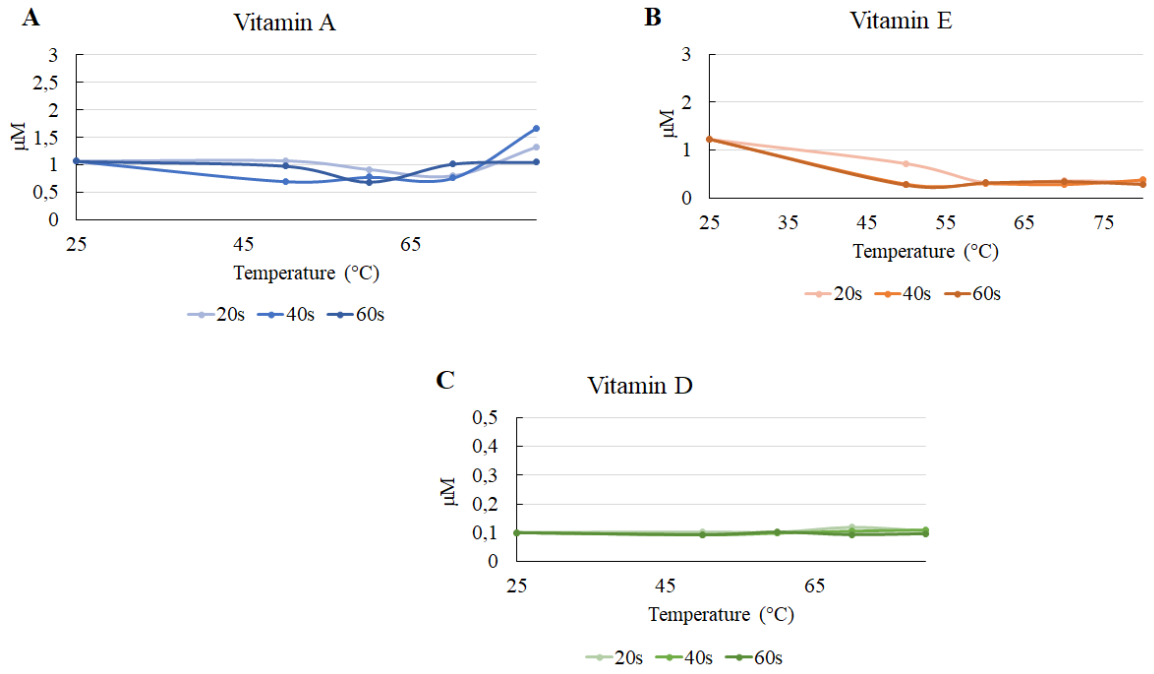


Figure 26. A) Vitamin A; B) Vitamin E; C) vitamin D content in HQ milk subjected to heat treatment (from 50 to 80°C for 20, 40, 60 seconds).

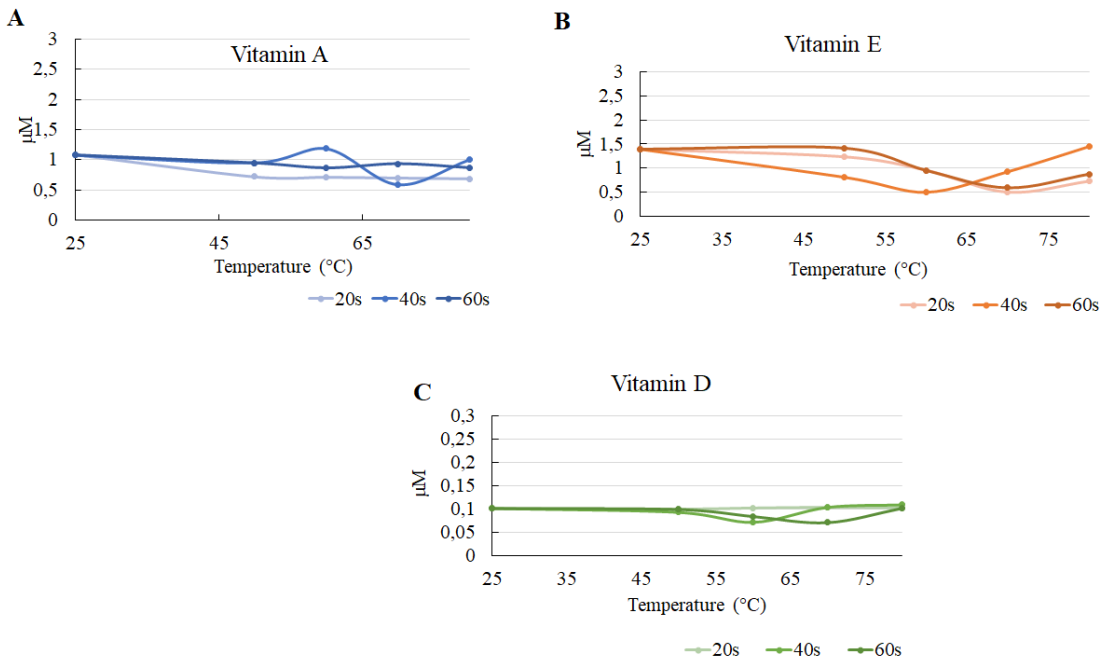


Figure 27. A) Vitamin A; B) Vitamin E; C) vitamin D content in HQ milk subjected to steam injection treatment (from 50 to 80°C for 20, 40, 60 seconds).

Vitamin A (retinol, retinoic acid, retinaldehyde) is an important nutrient for vision, tissue growth and differentiation, cell division, reproduction, and immunity. It is also known for its antioxidant properties. Vitamin E is a group of eight fat-soluble compounds that includes four tocopherols and four tocotrienols, the most biologically active compound being alpha tocopherol (α-tocopherol). Vitamin E has many functions, first of all, it has the role as a fat-soluble antioxidant since it acts as a free radical scavenger. Being fat-soluble, vitamin E is incorporated into cell membranes, which are thus protected from oxidative damage. Vitamin E influences gene expression and is a regulator of some enzymatic activities. Vitamin D refers to a group of fat-soluble secosteroids necessary for numerous biological functions, such as the homeostasis and metabolism of calcium and phosphate, promoting the physiological growth of the skeleton, bone remodeling and preventing degeneration in the elederly. A promoting effect of vitamin D on intestinal absorption of calcium, phosphate and magnesium is also observed. Nevertheless, the vitamin D group has an important role in cell growth, various neuromuscular and immune functions, and the reduction of inflammation.

5.5 Lipid Peroxidation Evaluation in Milk and Cappuccino

Lipid peroxidation is the degradation of lipids that occurs because of oxidative damage and therefore its evaluation is a useful marker for oxidative stress. Polyunsaturated lipids are in fact susceptible to an oxidative attack, typically by reactive oxygen species (ROS), resulting in the production of end products such as malondialdehyde (MDA). Other factors that can induce lipid peroxidation in foods are environmental factors that include heat, radiation, light, and food packaging (Custodio-Mendoza et al., 2022)

Therefore, the measurement of the MDA level in milk is directly proportional to the extent of lipid peroxidation (**Figure 28**). Lipid peroxidation products seem to be directly involved in the development of several pathologies such as atherosclerosis, cancer, and ageing processes (Burg et al., 2010). Several works evidenced that the formation of lipid peroxidation end products can have a role in promoting cancers and cardiovascular diseases (Ajmal et al., 2018). Fatty acid composition influences the rate of lipid peroxidation. In addition, the formation of the lipid peroxidation end products, have a detrimental effect also on milk flavour, can change the texture of the milk and can lead to the loss of its nutritive value.

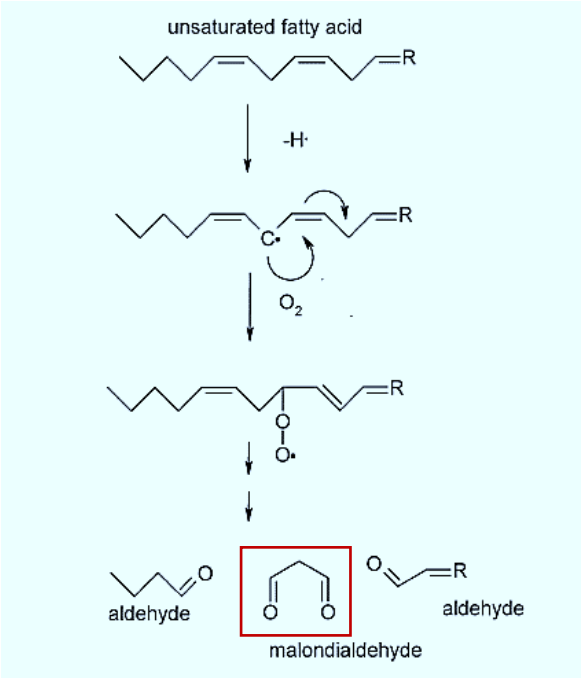


Figure 28. Lipid peroxidation and formation of malondialdehyde (MDA). From Burg et al., 2010.

In these experiments, the concentration of MDA has been evaluated in the HQ milk, in the coffee used for cappuccino preparation, and in HQ cappuccinos produced at increased temperature by the steam injection treatment. As discussed above, the greater the amount of MDA, the greater the rate of lipid peroxidation in the milk sample evaluated. The equation of the calibration line (**Figure 29**) obtained plotting the concentration of MDA versus the absorbance at λ= 532 nm resulted to be y=0.0959x+0.0744 (r^2=0.99), whereas the MDA content in HQ milk and HQ cappuccino samples after treatment with steam injection at different temperature is shown in **Figure 30**.

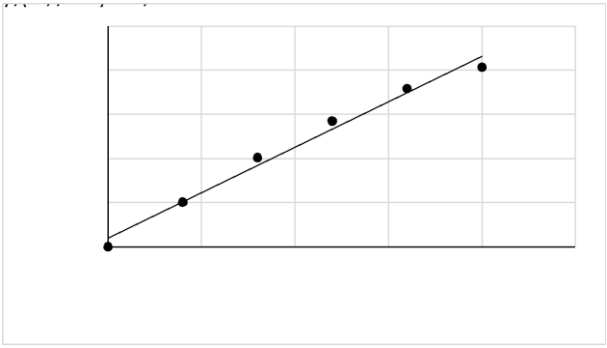


Figure 29. MDA standard calibration line

MDA content in HQ milk not heated was 60.17±0.2 nmol/ml, very similar to the value found by Kapusta and co-workers (2018) which was in the range between 48-86 nmol/ml.

If HQ milk is steam injected to the temperature of 60 °C, which is the one normally used in the production of cappuccino, the concentration of MDA was the same as the untreated milk (60.1 ±0.2 nmol/ml), and only at 80°C there was a significative decrement of MDA concentration (27.5±2.9 nmol/ml).

The content of MDA is significantly higher in the HQ cappuccino (139.9 ±9.9 nmol/ml) with respect to the HQ milk (without coffee) in the temperature range 60-80°C (**Figure 30**), indicating that the lipid peroxidation process is faster in cappuccino if compared to the same milk sample but without coffee. Lipid peroxidation has been also evaluated in the coffee sample used for the cappuccinos preparation and the content of MDA resulted to be 99.4±6.1 nmol/ml, and this could explain the highest MDA value found in the cappuccino samples with respect to the milk alone.

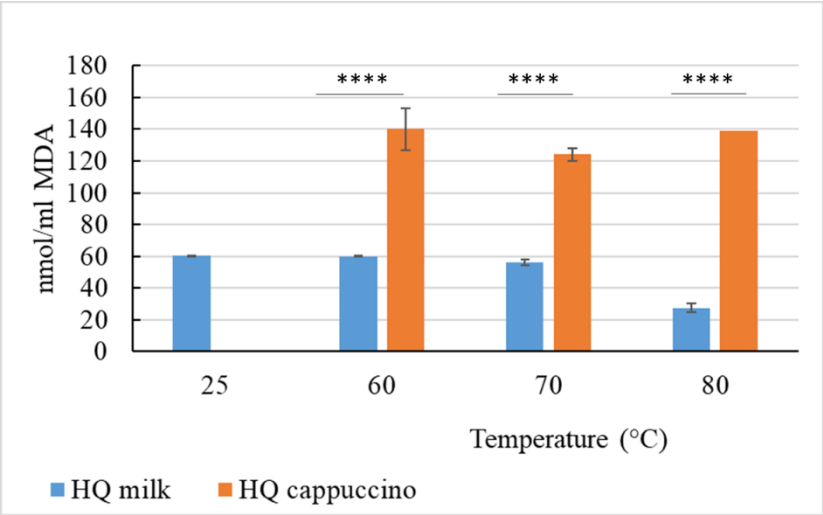


Figure 30. Behavior of MDA in HQ milk and HQ cappuccino after steam injection at different temperatures. * P<0.05; ** P<0.01; *** P<0.005; ****P<0.001.

On the other hand, the higher amount of MDA observed in coffee, may be due to the presence of hydrogen peroxide (H_2O_2) as recently described by Uppu and co-workers (2020). These authors measured H_2O_2 in freshly brewed coffee from different coffee companies using the ferrous oxidation-xylenol orange binding (FOX) assay and found that its content ranged between 0.29 and 0.82 mM, a value considered 5- to 20-fold higher than the concentration of H_2O_2 which is known to induce cytotoxic effects most mammalian cell types in culture.

Considering this evidence, in this work the concentration of H_2O_2 has been determined in the espresso blend used for cappuccino preparation (100% medium roasted Arabica coffee), using the enzymatic methods described in the Materials and Methods section.

In the first method, the H_2O_2 concentration (substrate) has been calculated through the Michaelis-Menten through the K_m (0.039 mM) and V_{max} (0.711 U/ml) values previously calculated by the Lineweaver-Burke plot. The concentration of H_2O_2 in freshly brewed espresso calculated with this first method was 25.9±1.44 µM.

In the spectrophotometric method of Cai and co-workers (2018) the hydrogen peroxide in freshly brewed espresso was determined as described in the Materials and Methods section. **Figure 31** shows the calibration line obtained by plotting the ΔA_{436} subtracted with the blank, versus increasing concentrations of standard H_2O_2 solutions.

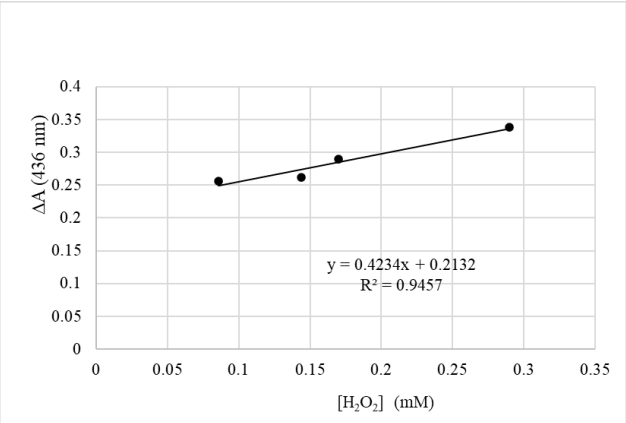


Figure 31. Calibration line for the measurement of H_2O_2 concentration in the coffee with the method of Cai et al. (2018).

In this case, the H_2O_2 concentration in freshly brewed espresso resulted to be 32.5±1.5 μM, very close to that obtained with the first determination method.

The amount of H_2O_2 found in the coffee blend used in this work is much lower than that found by Uppu and co-workers (2020) but is very similar to those (30-50 μM) found by Hiramoto et al. (2002). At this concentration level, the H_2O_2 in coffee should not have cytotoxic effects but could enhance the process of lipid peroxidation in coffee and consequently in the cappuccino.

From the literature, it is known that coffee has antioxidant properties due to the presence of phenolic compounds such as chlorogenic acids, caffeine, however the different preparation procedures of coffee can affect the antioxidant activity: it was found in fact, that when milk is added to the coffee, antioxidant activity decreased (Sánchez-González et al., 2005). The same authors demonstrated that the reduction of antioxidant activity was proportional to the amount of milk added.

This decrease may be because one of the most potent antioxidants in coffee is sequestered by the milk protein (especially β-lactoglobulin, α-lactalbumin, and lactoferrin) to form complexes. It is also known that heating enhances the formation of hydrophobic interactions involved in reversible polyphenol/protein interactions (Siebert et al, 1996; Dupas et al, 2014), whereas milk fat doesn't influence the amount of bound chlorogenic acids. Therefore, during cappuccino preparation, where the temperature of the beverage reaches 60-65°C, the above-mentioned hydrophobic interaction between milk protein and coffee phenolic compounds can increase, leading to further decrement of the antioxidant properties of the coffee.

5.6 Lactose Content Evaluation

Lactose, a disaccharide consisting of glucose and galactose, is one of the main constituents of milk and acts as an energy-carrier in milk. It is metabolized more slowly than glucose or sucrose, therefore lactose may be a good choice to reduce catabolite repression. Cow's milk typically contains about 4.8% lactose, and this value decreases with advancing lactation and mastitis infection. There is little effect of breed or individuality or nutrition of the animal on the lactose content of its milk.

In this work, lactose content has been determined on HQ milk (without coffee) and on cappuccino samples prepared with the HQ milk both treated with steam injection at increasing temperatures, as described in the Materials and Methods section. This determination was made in order to evaluate the effect of the increasing temperature induced by the stem injection on the content of this disaccharide and also if there were any interactions between the coffee compounds and lactose.

From **figure 32** it is evident that there is a decrease in the concentration of lactose among the HQ milk samples induced by the temperature ($P<0.005$ between HQ milk not treated and HQ milk at 60°C). There is no difference in the lactose concentration between HQ milk and HQ cappuccino, only in the samples treated at 80°C a slight decrement in lactose content can be observed induced by the temperature ($P<0.005$ between HQ milk and HQ cappuccino at 80°C).

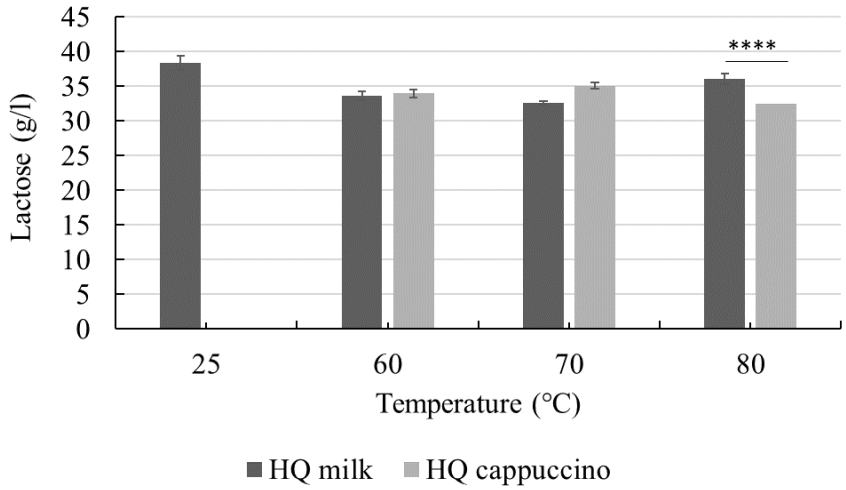


Figure 32. Lactose content (g/l) in HQ milk and in cappuccinos prepared with HQ milk, both treated at increasing temperature (up to 80°C) with the steam injection. * $P<0.05$; ** $P<0.01$; *** $P<0.005$; ****$P<0.001$.

5.7 In Vitro-Digestion Experiments of Individual Milk Proteins, HQ Milk and HQ Cappuccino.

The worldwide prevalence of food-related diseases has been increasing in recent decades. Large-scale human intervention trials have been used to correlate diet with the health of individuals demographic groups. However, to understand the physiological response to specific foods, it is necessary to follow the complex digestive processes within the human digestive tract in more detail. This can be achieved with invasive procedures, such as aspiration from the stomach or small intestine or with less invasive imaging technologies and wireless telemetry systems. Animal models are also widely used, although their use generally involves dead animals or surgical approaches in which cannulae are inserted into the digestive organs to access the contents of the gastrointestinal tract. The relevance of animal models for the understanding of food digestion in humans is also regularly questioned. In summary, *in vivo* intervention trials (human or animal) can be difficult to undertake, for inadequate and expensive or ethically unjustifiable reasons. In this regard, *in vitro* models have been used for many decades to simulate the digestion of food. (Brodkorb et al., 2019). There are several types of *in vitro* digestion methods commonly used for food; these can be divided into static and dynamic methods. These models aim to simulate physiological conditions of the upper gastrointestinal tract, the oral, gastric and small intestine phases. The most dynamic models have proven to be suitable for simulating the digestion of food and pharmaceutical products produced in different population groups and for different purposes. However, these models are relatively complex, expensive to set up and maintaining, and therefore may not be available for most of food researchers.

Thanks to its simplicity, static models, which use a constant ratio of food, enzymes and electrolytes and a constant pH for each digestive phase, have been used extensively for many decades for food, animal feed and pharmaceutical purposes. Static models of the *in vitro* digestion have been shown to be very useful in predicting digestion outcomes *in vivo*. However, a large number of digestion methods remain in use, reviewed by Hur et al., with slight but important changes in parameters such duration, activity and concentration of enzymes, pH, composition of the simulated digestive fluids. Thus, the need for the harmonization of digestion conditions was identified by the international INFOGEST network of multidisciplinary experts (in nutrition, enzymology, engineering, food science, and gastroenterology) from more than 35 countries. One of the primary results of this network was an international consensus on several digestion parameters for a static *in vitro* simulation of adult digestion suitable for food. (Brodkorb et al., 2019)

The digestion procedure can be summarized as shown in **Figure 33**, it can be divided into three phases: preparation, digestion procedure and sample treatment with subsequent analysis. (Brodkorb

et al., INFOGEST static *in vitro* simulation of gastrointestinal food digestion. Nat Protoc 14, 991–1014 (2019).

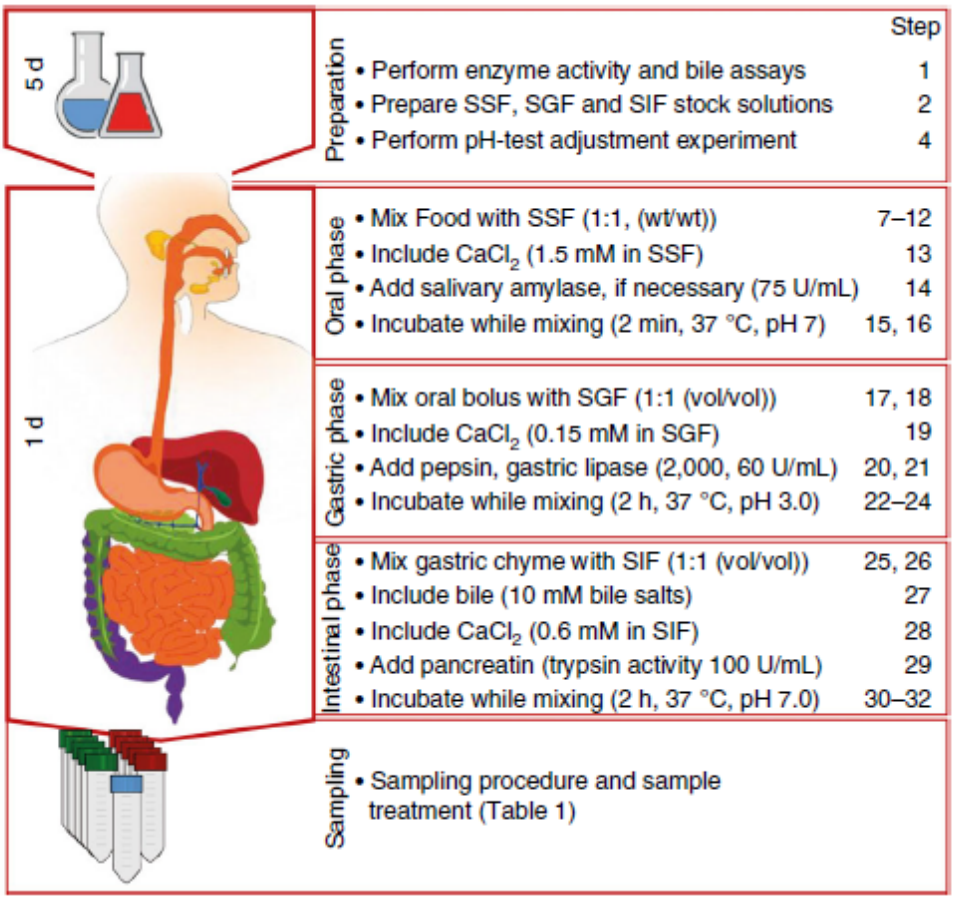


Figure 33. Flow diagram of the INFOGEST 2.0 digestion method. Timing and flow diagram of the INFOGEST 2.0 *in-vitro* digestion method for food. SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SSF, simulated salivary fluid. (From Brodkorb et al., 2019)

In Vitro Digestion of Individual Milk Proteins

From the electrophoretic analysis (15% SDS-PAGE) of digested β-lactoglobulin samples performed as described under the Materials and Methods section (*In vitro digestion of individual milk proteins*), β-lactoglobulin remains almost entirely intact in the gastric phase because at the acidic pH of the stomach this protein coagulates and forms a clot that makes almost impossible the action of gastric enzymes. The digestion of β-lactoglobulin starts in the intestinal phase, where pH rises to 7.0. At this pH, the protein structure is more relaxed, and the proteolytic intestinal enzymes can carry out their enzymatic activity. After two-hours of incubation in the intestinal phase, β-LG is, in fact, almost completely digested. (**Figure 34**).

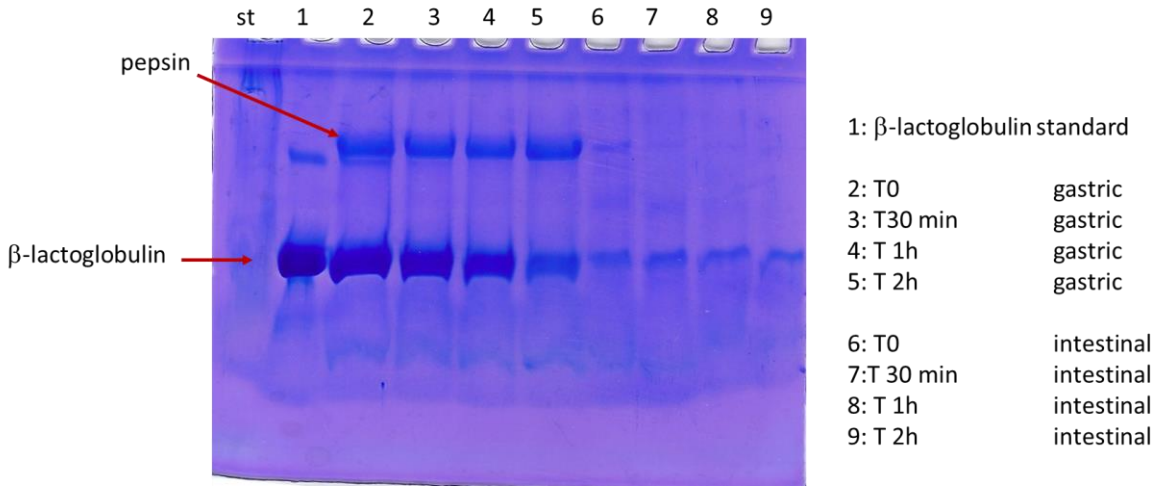


Figure 34. 15% SDS-PAGE showing the time course of the β-lactoglobulin gastrointestinal digestion. St: SDS-PAGE Molecular Weight Standards, Low Range (97.4-14.4 kDa).

These results are in agreement with what is reported in the literature, since the native β-LG is resistant to gastric digestion and apparently remains intact afterward passing through the stomach (Miranda & Pelissier, 1983; Yvon et al., 1984). However, the heat treatment could alter the digestibility and bioavailability of β-LG. The milk undergoes thermal treatments such as preheating, sterilization, dehydration, concentration, and other, which could affect its structure and properties reversibly or irreversibly. The reversible conformational change and denaturation of β-LG occurred below 70° C, but above this temperature, denaturation resulted in irreversible polymerization, as described in the introduction section (Dupont et al., 1965a, b; De Wit & Swinkels, 1980). At neutral pH, β-LG exists as a dimer but below pH 3.5 (which is the value found in the stomach) the dimer dissociates reversibly (McKenzie et al., 1971). This pH-induced dissociation is not accompanied by gross changes in molecular conformation (Timasheff et al., 1966; Mulini & Creamer, 1975; Teller et al., 1979). The acid stability of β-LG could derive from the increase of the internal hydrogen bond that is formed between two titrated carboxyl groups or an amide and a carboxyl group (Kella & Kinsella, 1988). Thus, the resistance of β-LG to peptic digestibility may reflect its stable conformation at pH 2.0. Pepsin has specificity for tryptophan, tyrosine, phenylalanine, leucine, and isoleucine (Fersht et al; 1977) and the resistance of the protein to peptic digestibility indicates that these groups are not accessible to the enzyme.

The behaviour of α-lactalbumin after the *in vitro* gastrointestinal digestion is shown in **Figure 35**. It is evident that this protein is more susceptible than β-lactoglobulin to gastric digestion. In fact, after

30 min incubation with the gastric phase, the protein is digested, and lower molecular weight peptides are produced. Therefore, once α-lactalbumin reaches the intestinal stage, it is completely digested.

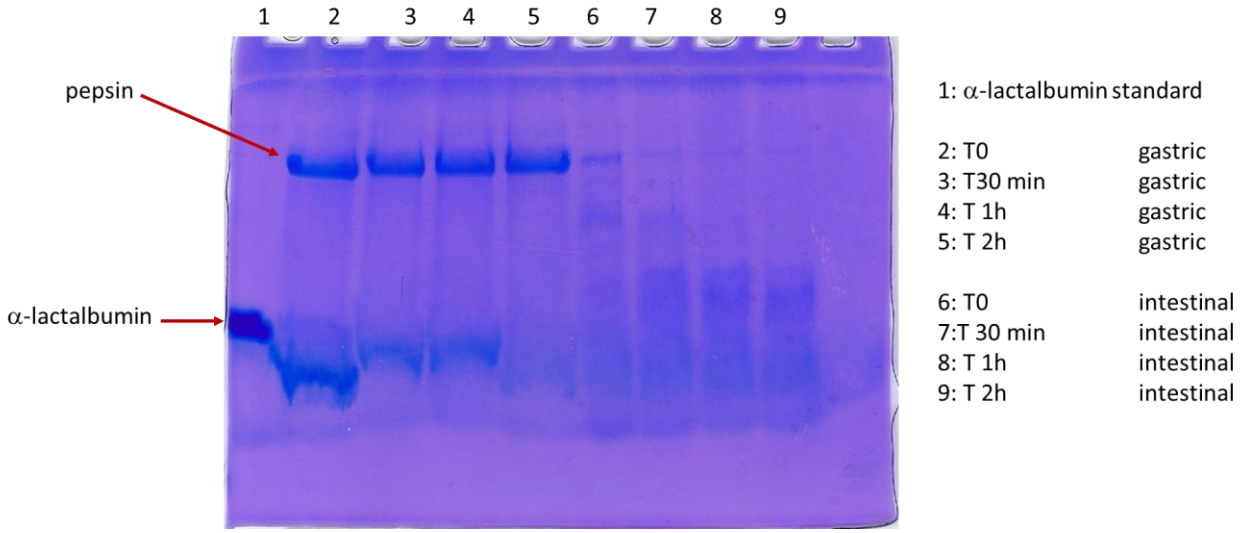


Figure 35. 15% SDS-PAGE showing the time course of the α-lactalbumin gastrointestinal digestion.

The tertiary structure of α-LA is very similar to that of lysozyme (Smith et al, 1987). It has been shown that α-LA is a metal ion binding protein (Hiraoka et al., 1980) with a strong bond to Ca (II) site (which can also bind Mg (II), K (I) and Na (I)). The binding of Ca (II) with α-LA causes a conformational change, which alters only slightly the main elements of the secondary structure (Dolgikh et al., 1981). On the other hand, the environment around some Trp and Tyr residues changes significantly, in fact, the removal of Ca (II) causes an increase both in the accessibility of Trp and Tyr residues to the solvent molecules. Also, several other experimental methods have shown that Ca (II) repression shifts α-LA to an intermediate state called the "molten globule" state, which possesses an intact secondary structure, but without an intact tertiary structure. A noteworthy feature of the α-LA structure is the acid conformational transition that occurs between pH 3 to 4, which is partly due to competition between Ca (II) and protons for the carboxyl side chains including the calcium-binding site (Permyakov et al., 1981b, 1985). At very acidic pH (<3), α-LA is also in the "molten globule" state (Dol gikh et al, 1981), which in some respects resembles the apo-conformation (Permyakov et al., 1981a, b, 1985). Even the binding of non-polar low molecular weight molecules in milk could also protect α-LA from digestion in the stomach. (Hirai et al; 1991). The interactions with food components can also alter the resistance of food proteins to digestion. The decomposition kinetics of α-LA was altered during gastrointestinal digestion *in vitro* from interactions with physiologically relevant levels of phosphatidylcholine (PC), a surfactant that is abundant both in milk and secreted from the stomach. The protective effect of PC was lost in subsequent duodenal digestion, with the α-

LA being rapidly broken down. This is probably because the bile salts in the duodenal digestion mix disrupt the vesicular structure of the PC as well as a possible mild detergent action on the α-LA peptides (Moreno et al., 2005).

A behaviour similar to that of β-lactoglobulin was also observed in the *in vitro* digestion of bovine serum albumin (BSA), which was digested considering a concentration of 0.4 mg/ml, the amount normally present in bovine milk (**Figure 36**).

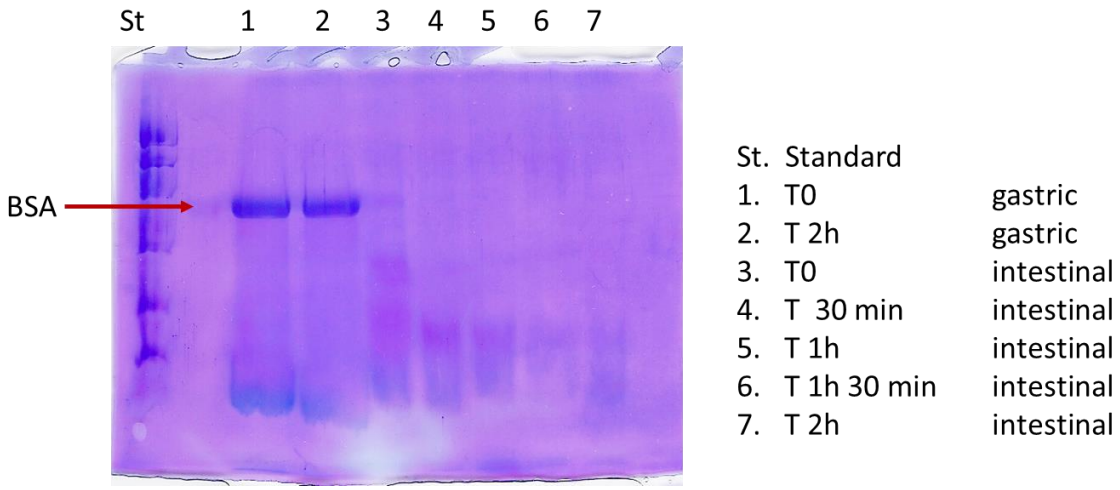
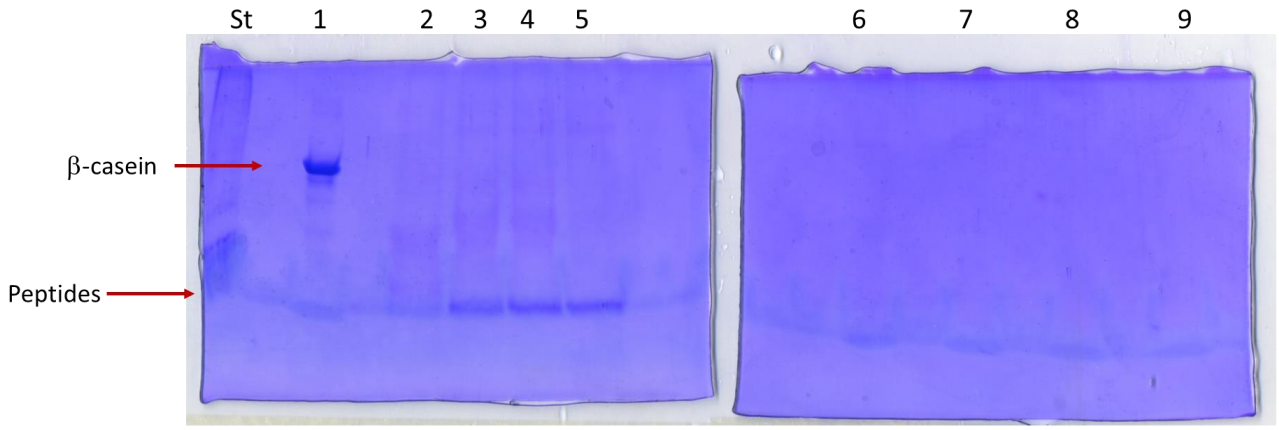


Figure 36. 15% SDS-PAGE showing the time course of the bovine serum albumin gastrointestinal digestion. St: SDS-PAGE Molecular Weight Standards, Low Range (97.4-14.4 kDa).

Bovine serum albumin is not digested during the gastric phase, the electrophoretic shows that protein band is still present after two-hours incubation with the gastric enzymes probably because of at pH 2.0 (gastric phase), BSA has a compact structure not accessible by the proteolytic enzymes. The protein is fully digested immediately as the intestinal phase begins. Also in this case, as for β-lactoglobulin, it can be assumed that the change in pH that occurs in the intestinal phase (pH 7.0) promotes the relaxation of the BSA structure thus allowing intestinal proteolytic enzymes to digest the protein (**Figure 36**).

Considering the digestion of β-casein (β-CN), it can be seen that this milk protein shows a completely different behaviour from that of the whey proteins observed so far (**Figure 37**).



St. Standard

1.	β-casein standard		6.	T0	intestinal
2.	T0	gastric	7.	T 30 min	intestinal
3.	T 30 min	gastric	8.	T 1h 30 min	intestinal
4.	T 1h 30 min	gastric	9.	T 2h	intestinal
5.	T 2h	gastric			

Figure 37. 15% SDS-PAGE showing the time course of the β-casein gastrointestinal digestion. St: SDS-PAGE Molecular Weight Standards, Low Range (97.4-14.4 kDa).

β-CN is completely and immediately digested during the gastric phase, after two-hour incubation with the gastric proteolytic enzyme the protein band corresponding to β-CN disappeared and low molecular weight peptides appear, resulting from the proteolytic digestion of β-CN. As also demonstrated in the literature, in gastric juice this protein undergoes degradation by pepsin. Pepsin shows a preferential cleavage for hydrophobic, preferably aromatic, residues. The degradation of β-CN begins at the hydrophobic C-terminal region of this amphiphilic protein with "soap-like" properties. (Schmelzer et al., 2007) It is known from recent studies that removing the C-terminal part AA 192 to 209 does not only affect aggregation behaviour but also has a strong destabilizing effect on the whole molecule. (Qi et al., 2005)

In Vitro Digestion of HQ Milk and HQ Cappuccino

After analyzing the digestive process of the individual milk proteins, the *in vitro* digestion of HQ milk (without coffee) and HQ cappuccino samples has been carried out, in order to find any difference in the digestibility of milk due to both the steam injection treatment (by the coffee machine Simonelli Group) and to the presence of the coffee compounds that may interact with the milk proteins.

In these experiments high quality pasteurized fresh milk (HQ milk) has been used and the milk samples and cappuccino samples were prepared before *in vitro* digestion experiments as described in the section "*Sample preparation*". The *in vitro* gastrointestinal digestion has been carried out as described in the "In *vitro digestion of HQ milk and HQ cappuccino samples"* section.

In **Figures 38** and **39** are shown the digestibility of HQ milk and HQ cappuccino, respectively. In both samples the protein band corresponding to caseins completely disappeared as soon as the gastric digestion starts, however it seems that the whey protein fraction is more digestible in the HQ cappuccino sample than in the HQ milk (without coffee).

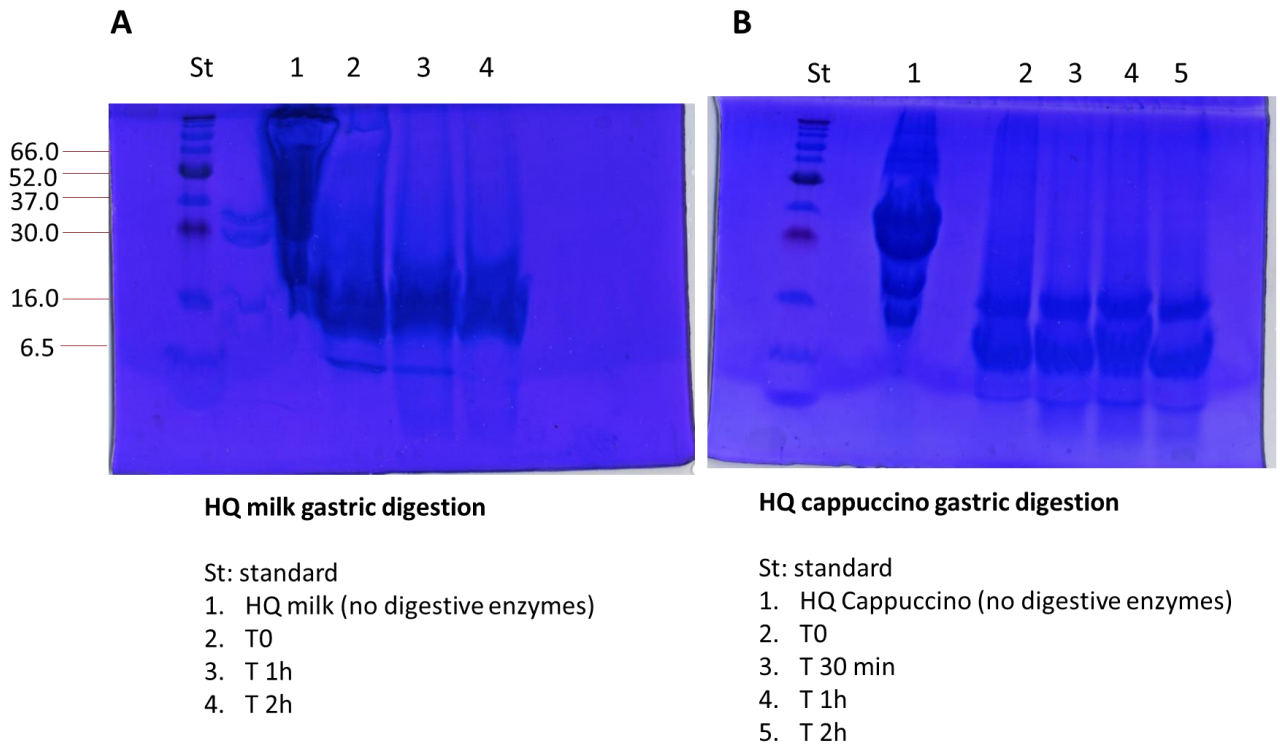


Figure 38. 15% SDS-PAGE showing the time course of HQ milk (**A**) and HQ cappuccino (**B**) gastric digestion. **St**: Prestained Protein SHARPMASS™ VII Protein MW marker (6,5-270 kDa).

Once in the intestinal phase both samples (HQ milk and HQ cappuccino) resulted completely digested by the intestinal proteolytic enzymes, as shown in **Figure 39.**

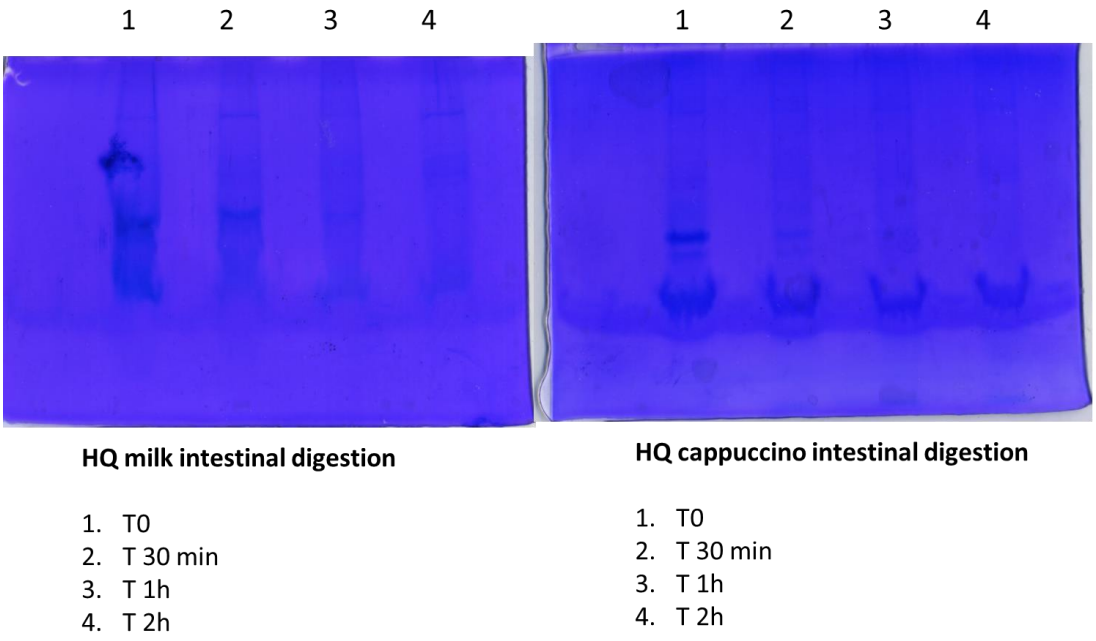


Figure 39. 15% SDS-PAGE showing the time course of HQ milk (**A**) and HQ cappuccino (**B**) intestinal digestion.

The greater digestibility of HQ cappuccino during the gastric phase compared to HQ milk could be due to the heat treatment to which the milk is subjected during the preparation of the cappuccino (60°C for 20-30 seconds) which could lead to a change of the whey protein structures and consequently to an easier accessibility of the proteolytic enzymes or may be due to the formation of complexes between milk proteins and coffee compounds that could have a similar effect, or to a combination of both factors.

5.8 OPA-Assay Analysis

The evaluation of the gastrointestinal digestion products was carried out, as well as through the electrophoretic analysis previously shown, also through an OPA assay. Thanks to this assay it is possible to quantify the degree of hydrolysis (DH) which is a useful way to monitor the extent of protein degradation that occurs in the gastrointestinal digestion process. In particular, DH is defined as the percentage of the total number of peptide bonds in a protein that have been broken down during hydrolysis (Adler-Nissen, 1986). The test is based on the reaction of o-phthalaldehyde (OPA) and 2-

mercaptoethanol with amino groups released during the proteolysis of a protein substrate (for example during gastrointestinal digestion). For these reasons, to confirm the results obtained by the electrophoretic analysis and to get a more complete framework of digestibility of the HQ milk and HQ cappuccino, after the *in vitro* digestion these samples were subjected to the DH analysis by OPA-assay (Church et al., 1984). In addition to the HQ milk sample not heated (not subjected to steam injection treatment), and the HQ cappuccino (prepared at a temperature of 60°C) in these experiments was also evaluated the behavior of HQ milk subjected to steam injection at 60°C but without coffee (HQ 60°C), in order to evaluate whether the greater digestibility of HQ cappuccino compared to HQ milk observed in gastric digestion (see **Figure 40**) was due to the heat treatment that occur during the cappuccino preparation, to the formation of complexes between milk proteins and coffee compounds or both. The results obtained in these experiments showed that the DH is very low for all the three samples at the end of the gastric phase, indicating that the proteolytic process during the digestion phase is not complete (**Figure 40**). Different is the situation in the case of the intestinal digestion: both HQ milk treated with stem injection up to 60°C and HQ cappuccino (prepared at 60°C), had a significantly higher degree of protein hydrolysis than the HQ milk not heated after the intestinal digestion (**Figure 40**).

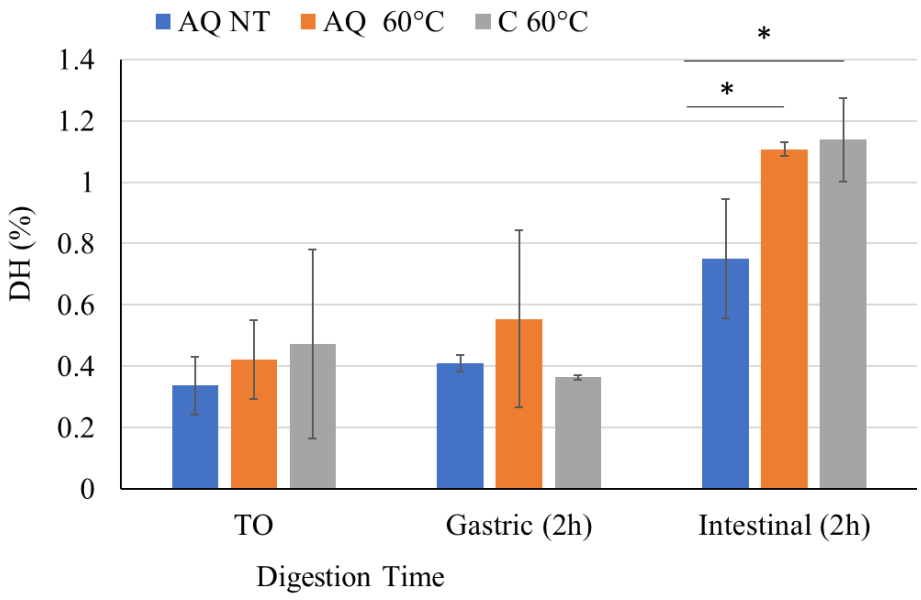


Figure 40. Degree of protein hydrolysis (DH) in the samples of HQ milk not heated (HQ NT), HQ milk treated with steam injection up to 60°C (HQ 60°C) and HQ cappuccino (C 60°C) assessed by

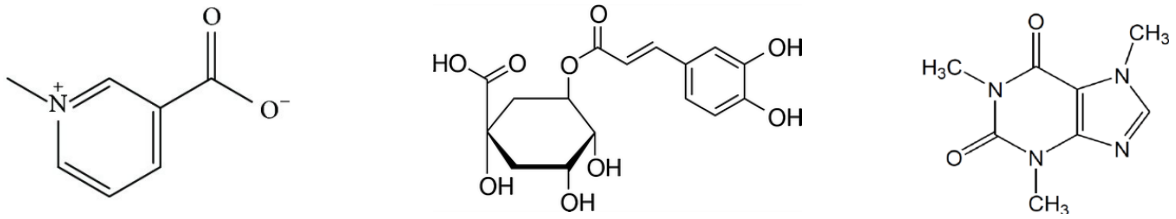
the OPA assay. Each sample was analyzed in the different digestion phases: at time T0, at the end of gastric digestion (Gastric 2h) and at the end of intestinal digestion (Intestinal 2h).

This result could indicate a greater digestibility of the HQ milk treated at 60°C with the steam injection and of the HQ cappuccino compared to the HQ milk untreated ($P<0.05$) and that most likely the responsible for this greater digestibility is only the effect of the treatment at 60°C with the steam injection, since there are no significant differences between the HQ milk treated at 60 °C and the HQ cappuccino.

5.9 Evaluation of the Binding Affinity Between the Main Milk Proteins and Coffee Compounds by Fluorescence Spectroscopy Studies

As mentioned in the "*Introduction*" chapter, in relation to cappuccino and the mixture of milk and coffee, it is very interesting to understand if there can be interactions between the main compounds of milk and those of coffee. This is important because the possible formation of complexes between the molecules of the two matrices could lead to changes in the behavior of the molecules involved, in particular with regard to digestibility, bioavailability and therefore their effect on our organism. Milk proteins (caseins and whey proteins) have functional properties that give the final product a structural edge in terms of emulsifying and foaming capacities, among their unique interfacial properties. However, both caseins and whey proteins can interact with other food components, such as fats, sugars, polysaccharides, salts, flavors, aromas compounds and bioactives (for example phenolic compounds), which can alter their thickening, gelling and flavor-binding properties, depending on the processing conditions. In addition, any change at the molecular level causing total or partial proteins unfolding (due to heat impact) can also have an influence on functionality and reactivity, with unpredictable outcomes. Changes in the three-dimensional structure of milk protein can determine the type and strength of various interactions, the interfacial structure, and other characteristics, like texture, rheology, sensory properties and shelf life of final products (Raikos, 2010; Singh & Ye 2020). It is important to note that strong associations between milk proteins and polyphenolic compounds of vegetable origin have been reported; such interactions may not only diminish the activity of these compounds but also the functionality of milk proteins. (Rashidinejad et al., 2017)

The tested milk proteins were β-lactoglobulin (β-LG), α-lactalbumin (α-LA), β-casein (β-CN), and lactoferrin. At the same time, the tested coffee molecules were caffeine, chlorogenic acid, and trigonelline, showed in **Figure 41**.



Trigonelline **Chlorogenic acid** **Caffeine**

Figure 41. Chemical structure of Trigonelline (From Nuhu, 2014), Chlorogenic acid (From Kamiloglu et al., 2018) and Caffeine (From Belay, 2011).

Fluorescence spectroscopy analyses were carried out on the protein samples, exploiting the emissive properties of the aromatic amino acid residues, such as tryptophan and tyrosine. For each protein, once a concentration, resulting in a suitable spectrum was established, gradual additions of the coffee compound were made up to observe the phenomenon of quenching of emission of the protein.

B-Lactoglobulin (B-LG) Binding Test

The following **Figures 42**, **43**, respectively show the emission spectrum of the β-LG in the binding affinity tests carried out with chlorogenic acid and the relative dissociation constant.

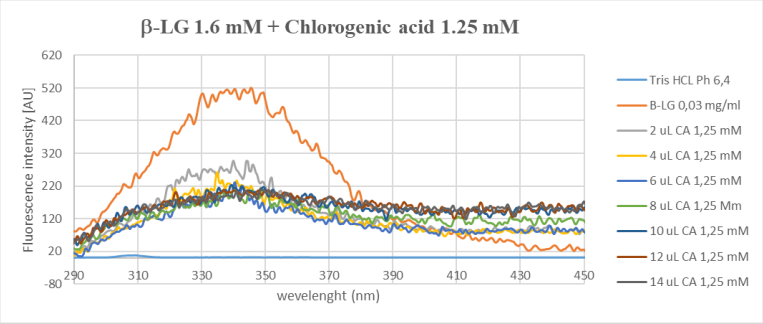
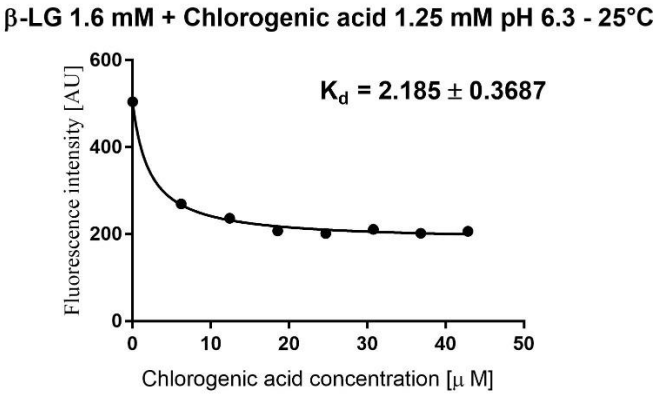


Figure 42: Spectra obtained from the fluorescence analysis of the binding affinity between β-LG and chlorogenic acid. The emission intensities were corrected taking into account protein dilution due to chlorogenic acid additions.

Figure 43: Dissociation constant (K_d) calculated from the binding experiment, determined by fitting the experimental data using the equation 1.



In this case it has been used a concentration of β-LG of 1,6 mM, to which a total concentration of chlorogenic acid equal to 42.85 μM was added progressively. The binding process of chlorogenic acid causes a red-shift in the emission of tryptophan indicating an increase in exposure to the solvent and therefore a conformational change of the protein that can be given by the interaction with chlorogenic acid. This can also be seen from the K_d value of 2.185 ± 0.37 μM, indicating a high degree of affinity between the two molecules.

The following **Figure 44**, **45** respectively show the emission spectrum of the β-LG in the binding affinity tests carried out with trigonelline and the relative dissociation constant.

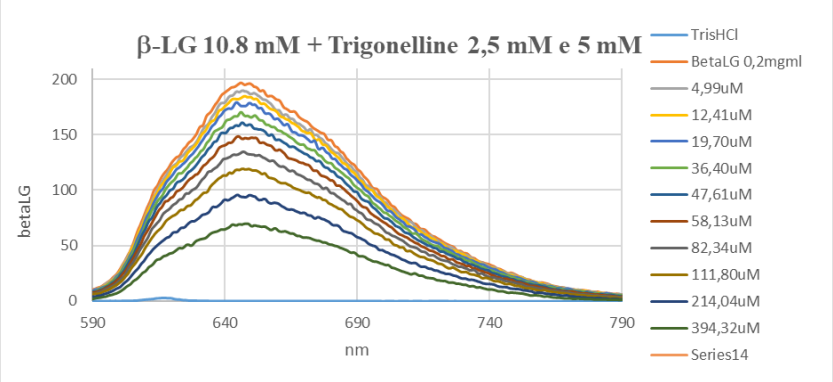
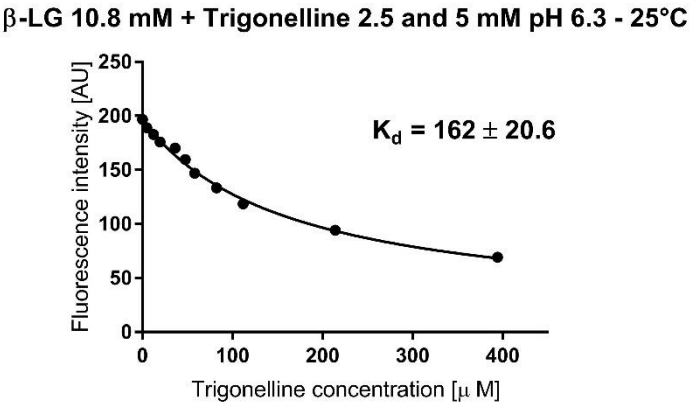


Figure 44: Spectra obtained from the fluorescence analysis of the binding affinity between β-LG and trigonelline. The emission intensities were corrected taking into account protein dilution due to chlorogenic acid additions.

Figure 45: Dissociation constant (K_d) calculated from the binding experiment, determined by fitting the experimental data using the software GraphPad Prism 7.



The concentration used of β-LG was of 10.8 mM to which a total concentration of trigonelline equal to 394 μM was added progressively. In this case a collisional quenching phenomenon has been observed, that can be explained by the fact that trigonelline is a polar and zwitteronic molecule, unable to efficiently interact with the β-LG hydrophobic pocket, but only with the most exposed tryptophan residue, without giving a real binding phenomenon.

These following **Figures 46, 47** instead, respectively show the emission spectrum of the β-LG in the binding affinity tests carried out with Caffeine and the relative dissociation constant.

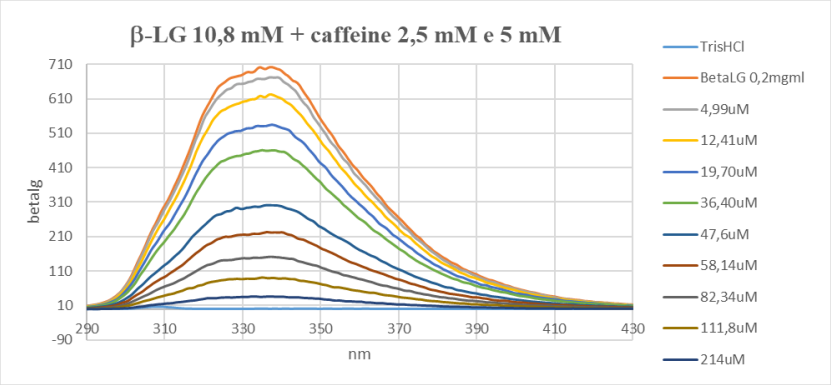
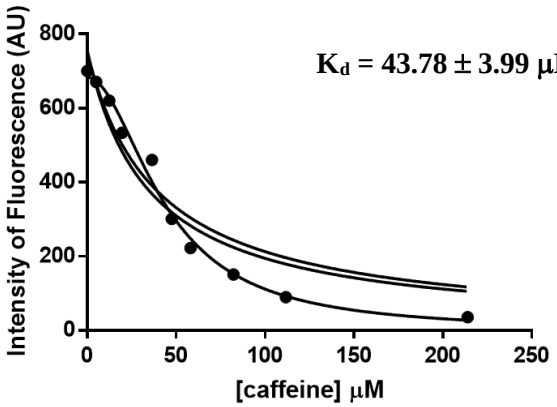


Figure 46: Spectra obtained from the fluorescence analysis of the binding affinity between β-LG and Caffeine. The emission intensities were corrected taking into account protein dilution due to caffeine additions.

Figure 47: Dissociation constant (K_d) calculated from the binding experiment, determined by fitting the experimental data using the software GraphPad Prism 7.



The concentration of β-LG was of 10.8 mM to which a total concentration of caffeine equal to 214 μM was added progressively. From the fitting of the experimental data obtained plotting the change in the intensity of β-LG intrinsic fluorescence induced by caffeine, it is possible to notice a higher dissociation constant than that for chlorogenic acid, indicating a lower binding affinity between the two molecules.

β-Casein (β-CN) Binding Test

In this case the following **Figures 48** and **49** respectively show the emission spectrum of the β-CN in the binding affinity tests carried out with chlorogenic acid and the relative dissociation constant.

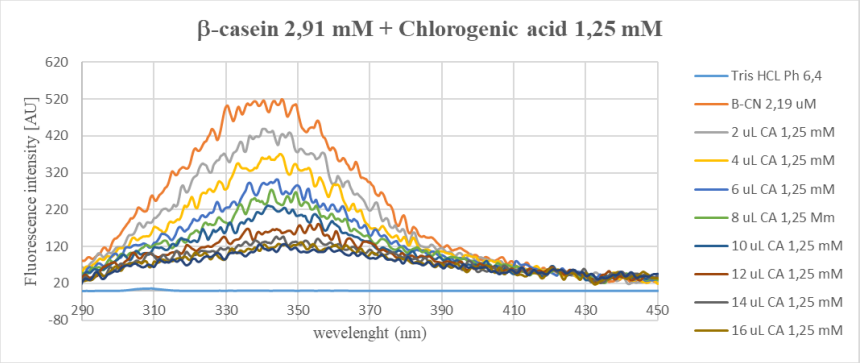
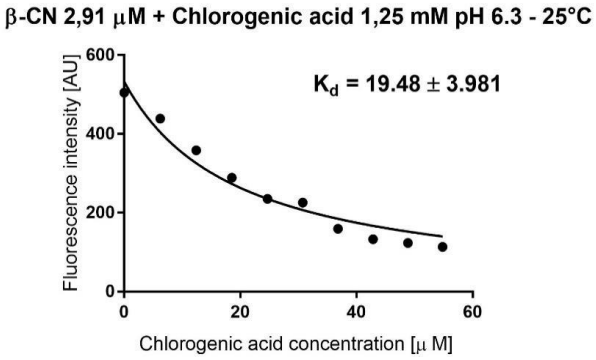


Figure 48: Spectra obtained from the fluorescence analysis of the binding affinity between β-CN and Chlorogenic acid. The emission intensities were corrected taking into account protein dilution due to chlorogenic acid additions.

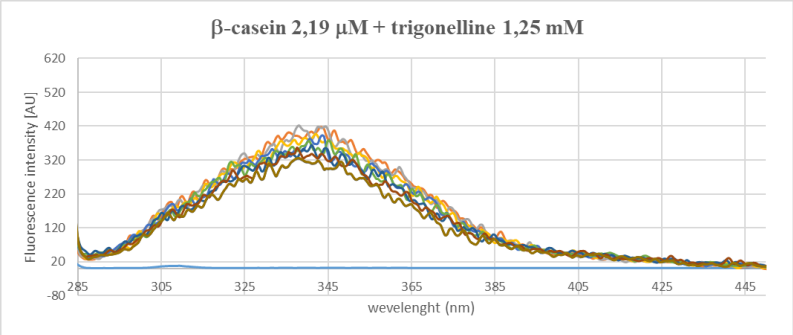
Figure 49: Dissociation constant (K_d) calculated from the binding experiment, determined by fitting the experimental data using the software GraphPad Prism 7.



For β-CN, in all tests a concentration of 2.91 mM was used and the total concentration of chlorogenic acid added was equal to 48.85 μM. As can be seen in the fluorescence spectrum, the binding process of chlorogenic acid with β-CN in this case slightly causes a red-shift of the maximum of intensity (from ~340 to ~345 nm) indicating an increase in exposure to the solvent and therefore a conformational change of the protein. From the rather low K_d value, it is possible to infer a certain affinity for chlorogenic acid by β-CN.

The following **Figures 50** and **51**, respectively show the emission spectrum of the β-CN in the binding affinity tests carried out with Trigonelline and the relative dissociation constant.

Figure 50: Spectra obtained from the fluorescence analysis of the binding affinity between β-CN and Trigonelline. The emission intensities were corrected taking into account protein dilution due to trigonelline additions.



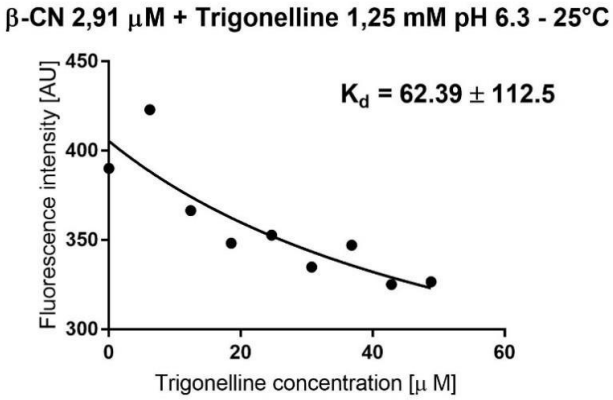


Figure 51: Dissociation constant (K_d) calculated from the binding experiment, determined by fitting the experimental data using the software GraphPad Prism 7.

In this case the total trigonelline concentration added on the β-CN solution was 48.85 µM, and the spectra obtained by the fluorescence analyses show a moderate quenching phenomenon, resulting in a high K_d, accounting for a low affinity between the two molecules.

The last following **Figures 52** and **53** regarding β-CN, respectively show the emission spectrum in the binding affinity tests carried out with Caffeine and the relative dissociation constant.

Figure 52: Spectra obtained from the fluorescence analysis of the binding affinity between β-CN and Caffeine. The emission intensities were corrected taking into account protein dilution due to caffeine additions.

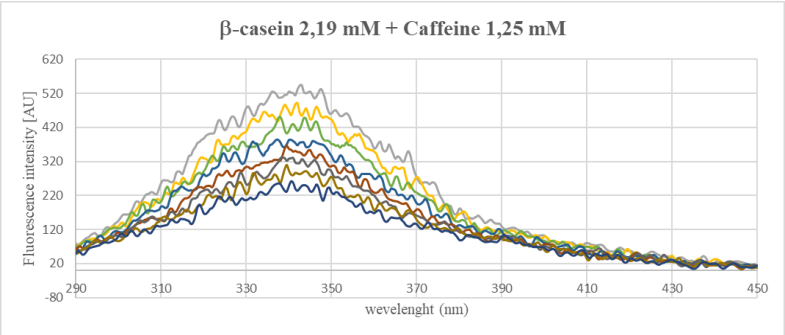
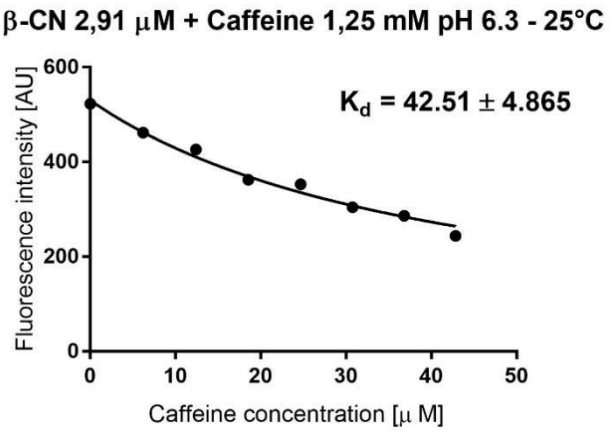


Figure 53: Dissociation constant (K_d) calculated from the binding experiments, determined by fitting the experimental data using the software GraphPad Prism 7.



Regarding caffeine, the total concentration added to the protein solution was 42.85 µM. In this case, considering the value of the dissociation constant, very similar to the one obtained for β-LG, it is possible to assess that the behaviour of these two proteins, in terms of binding affinity, is very similar towards these compounds present in coffee.

α-LA and Lactoferrin Binding Tests

Considering the previous results, for α-LA and lactoferrin, only the affinity tests with chlorogenic acid will be shown (**Figure 54, 55**), considering the same poor affinity with caffeine e trigonelline. In this case, the α-LA concentration was equal to 4.24 µM and the total concentration of Chlorogenic acid added was 54.83 µM.

Figure 54: Spectra obtained from the fluorescence analysis of the binding affinity between α-LA and Chlorogenic acid. The emission intensities were corrected taking into account protein dilution due to chlorogenic acid additions.

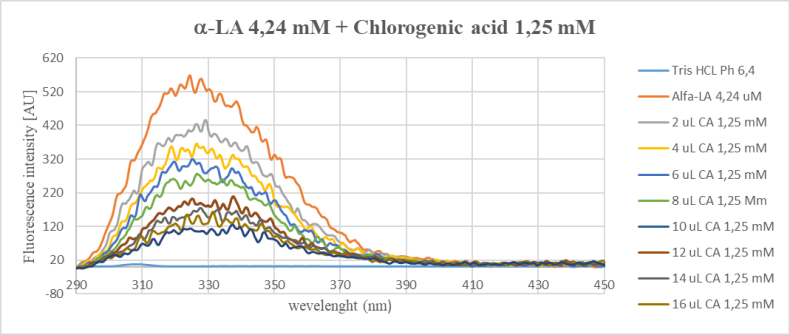
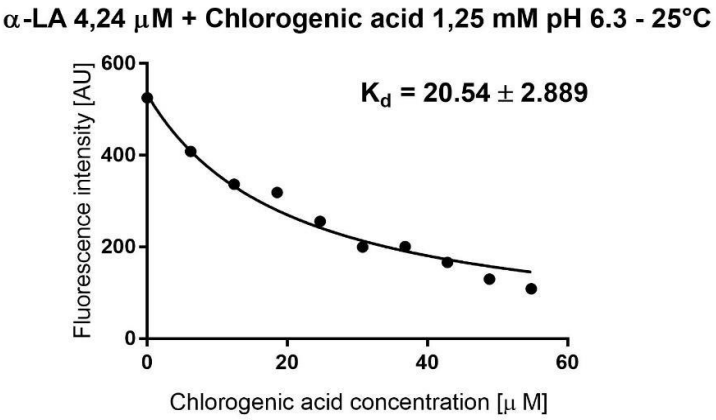


Figure 55: Dissociation constant (K_d) calculated from the binding experiments, determined by fitting the experimental data using the software GraphPad Prism 7.



The following **Figures 56** and **57**, respectively show the emission spectrum of Lactoferrin with a concentration of 0.38 mM, in the binding affinity tests carried out with Chlorogenic acid, with total concentration added of 30.86 μM, and the relative dissociation constant.

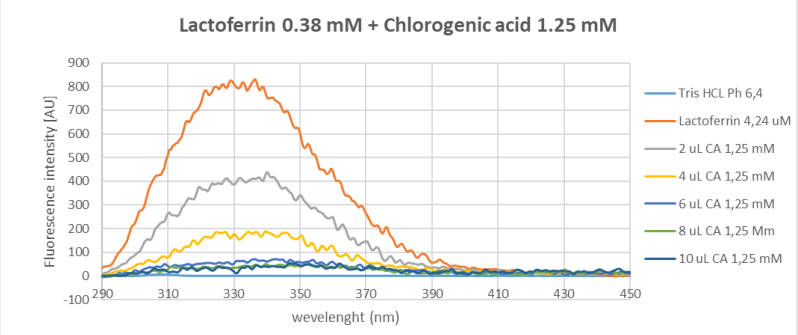
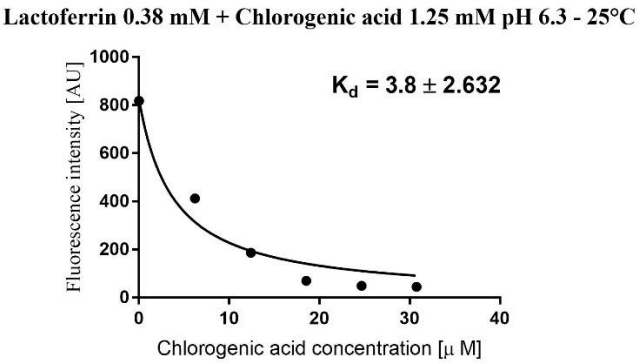


Figure 56: Spectra obtained from the fluorescence analysis of the binding affinity between Lactoferrin and Chlorogenic acid. The emission intensities were corrected taking into account protein dilution due to chlorogenic acid additions.

Figure 57: Dissociation constant (K_d) calculated from the binding experiments, determined by fitting the experimental data using the software GraphPad Prism 7.



In these last tests, it can be seen that for both whey proteins the spectral change induced by chlorogenic acid is characterized by a red shift with a consequent change in the conformation of the protein, in particular as regards lactoferrin which has a very low K_d.

Previous studies have demonstrated that milk proteins can interact with phenolic compounds in foods, which can alter nutrition, stability, taste, and colour (Li et al., 2020). For example, it has been reported that phenolic acids can interact with various types of milk proteins including whey proteins (Jiang et al., 2018), α-lactalbumin (Zhang et al., 2014), β- lactoglobulin (Wu et al., 2018), bovine serum albumin (Yuan et al., 2019) and casein (Kaur et al., 2018) forming phenolic acid-protein complexes. Interactions between dairy proteins and polyphenols have been reported that could modify the structure of proteins as well as their physical-chemical characteristics, such as proteins solubility, emulsifying and foaming properties, and also the digestibility (Dai et al., 2019; Jiang et al., 2018; Ozdal et al., 2013). Concerning β-casein, this protein contains both hydrophilic and hydrophobic regions in its polypeptide chain, making it an amphiphilic molecule. The unique structural organization of β-casein molecules makes them particularly suitable for the development of natural nano-delivery vehicles for hydrophobic and amphiphilic compounds (He et al., 2016; He et al., 2016).

Previous studies reported that polyphenols have relatively high binding affinities with β-casein and can be supplied and protected by this protein, including resveratrol (Acharya et al., 2013), naringenin (Moeiniafshari et al., 2015), quercetin (Ghayour et al., 2019), p-coumaric acid (Kaur et al., 2018) and tea polyphenols (Chanphai et al., 2018). The carrying ability of these proteins is however affected by the structure of the phenolic acid, and the binding affinity between the two molecules (Yildirim et al., 2018). As an example, Wu et al. (2018) reported that the position of hydroxylation, methylation and methoxylation of the phenyl ring affected the binding affinity of phenolic acids with β-lactoglobulin. Yuan et al. (2019) indicated that the structure of phenolic acid (including hydroxylation and methoxylation of hydroxyl groups) affected the ability to bind to bovine serum proteins. Regarding β-casein in general there is little information on its ability to interact with phenolic acids (Li et al., 2020). The present study evidenced the ability of β-casein to bind to coffee compounds, including chlorogenic acid, this is in agreement with previous studies which evidenced the binding between this protein and some coffee compounds, as shown in **Table 9.**

Table 9. K_d values obtained from the binding tests between the main milk proteins and some coffee compounds by fluorescence spectroscopy analysis.

TESTED PROTEINS	K_d for Chlorogein Acid	K_d for Caffeine	K_d for Trigonelline
β-lactoglobulin	2.185 ± 0.3687 μM	43.78 ± 3.99 μM	-
β-casein	19.48 ± 3.981 μM	42.51 ± 4.865 μM	62.39 ± 112.5 μM
α-lactalbumin	20.54 ± 2.889 μM	-	-
Lactoferrin	3.8 ± 2.632 μM	-	-

All the proteins analyzed showed similar behaviour, with a high affinity for chlorogenic acid, in particular β-LG and lactoferrin, while all showed a low affinity for caffeine and trigonelline. This aspect could be very interesting in the context of the study of cappuccino, because the possible formation of complexes between the milk proteins and the chlorogenic acid from coffee could lead, as previously mentioned, to changes in behaviour for both molecules in terms of digestibility and bioavailability. Further studies will be designed to evaluate how the formation of high-affinity complexes between chlorogenic acid and milk proteins can affect cappuccino properties, including digestibility.

6. CONCLUSIONS

This work aimed to characterize, from the molecular, nutritional, and digestibility point of view, cappuccinos prepared using the Simonelli Group coffee machines under different steam injection conditions. The milk used in these experiments was High-Quality pasteurized milk (HQ milk, the type of milk most used by barmen for the preparation of cappuccino) and for some experiments was also used UHT milk, for a comparison with the pasteurized milk.

The study on the protein profile of cappuccino and relative foam, performed on both HQ and UHT milk, confirmed the previous studies carried out by other authors on the milk protein ability to form a foam, highlighting that the β-casein and genetic variants of β-lactoglobulin, but also other whey proteins such as lactoferrin, may play a very important role in the foam formation and stability. In **Figure 58**, a speculative schematic representation of the milk protein absorbed at the interface air-liquid is represented. It is known that the predominant class of casein responsible for the surface properties is β-casein (Marinova et al., 2009) which, as discussed before, is less structured of the α-caseins. β-casein, in fact, possesses a large hydrophobic region 159/209 amino acids that are extended as an inner dense layer of 1-2.5 mm whereas the hydrophilic region (about 50 amino acids), is facing outwards constituting a less dense layer of 3-7.5 mm forming a "tail" or a "loop". Since caseins are resistant to temperature, the increasing temperature induced by the steamer during cappuccino preparation do not affect casein's concentration even at 80° for 60 seconds, therefore no change in the distribution of caseins in the adsorption layer is expected, which remains dense and thick ensuring the foam stabilization. On the other hand, globular whey protein adsorbs almost intact at the interface, however at increasing temperature induced by the stem injection, β-lactoglobulin and lactoferrin concentration decreases in the cappuccino foam samples indicating that these proteins could form aggregates that are trapped in the films. α-lactalbumin concentration is not affected by the increasing temperature and, probably, could not compact well to ensure the necessary film and foams stabilization.

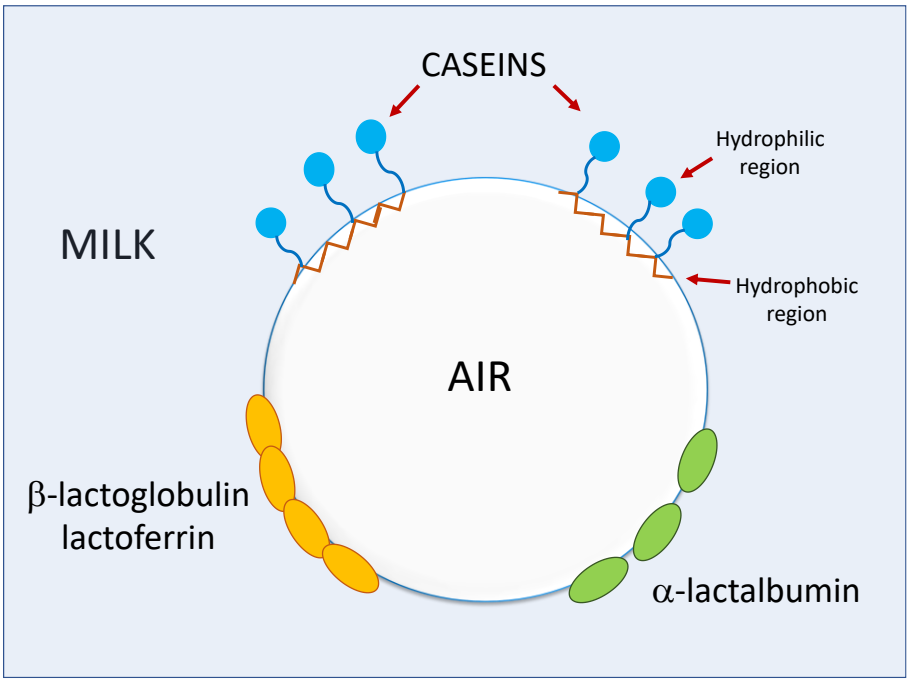


Figure 58. Model representing the hypothetical distribution of caseins and whey proteins absorbed at liquid/air surface.

Lactoperoxidase, an enzyme which is usually monitored in the milk because of its antimicrobial property, showed an unaltered enzymatic activity up to 70°C then at high temperatures (75-80°C), the enzymatic activity began to decrease significantly but in the latter case, these are temperatures that are never reached neither in the preparation of the cappuccino in cup nor in that of the take-away cappuccino.

The effect of increasing temperature and steam injection treatment on the water- and fat-soluble vitamins of HQ milk has been studied, in order to evaluate whether the concentration of vitamins was dependent only on the increase in temperature, the steam injection, or both. The results showed that the content of some water-soluble vitamins, nicotinic acid, nicotinamide, vitamin B6, vitamin B2, and folic acid was not influenced by the increase in temperature alone (temperature range 50-80°C for 20-60 seconds). However, the treatment with steam injection at the same temperature and exposure times led to a decrease of vitamin B6 and folic acid at a temperature higher than 70°C and a substantial decrease in nicotinamide content already at 60°C but, at the same time, an increase in nicotinic acid was observed. The increase of this latter form of vitamin B3 could derive from the deamidation of nicotinamide induced by the steam injection process, however further studies will be needed to validate this hypothesis or to find alternative reasons for this result. The content of fat-soluble vitamins in HQ milk is not influenced by heat treatment alone, nor by steam injection, with the exception of vitamin E which seems affected only by heat treatment.

The lactose content was not affected by the temperature increase induced by the steam injection, both in the HQ milk and in the HQ cappuccino. Only at a temperature of 80°C is there a significant decrease in the lactose content in the HQ cappuccino compared to the HQ milk sample.
In light of these results, it can therefore be stated that the treatment with steam injection at a temperature of 60-65° C (which is normally the one used for cappuccino preparation) did not affect the content of the water- and fat-soluble vitamins and lactose in the HQ milk, thus maintaining its characteristics intact from the nutritional point of view.

Lipid peroxidation is the degradation of lipids that occurs because of oxidative damage and therefore its evaluation is a useful marker for oxidative stress. Typically, it is caused by reactive oxygen species (ROS), resulting in the production of end products such as malondialdehyde (MDA) which has several negative effects on human health. The lipid peroxidation evaluation on HQ milk and HQ cappuccino, showed a significantly higher amount of MDA in the HQ cappuccino with respect to the HQ milk in all temperature ranges (from 60° to 80°C), indicating that the lipid peroxidation process is faster in cappuccino than in milk without coffee. In this work it was found the presence of hydrogen peroxide (H_2O_2) in the coffee used in the cappuccino preparation in amounts not harmful to human health but that could induce the lipid peroxidation process observed in cappuccino samples.

The digestibility of single milk proteins such as β-lactoglobulin, α-lactalbumin, β-casein, and bovine serum albumin as well as of HQ milk and HQ cappuccino has been evaluated through an *in vitro* digestion protocol to find any differences due or to the different digestibility of the single milk proteins or of complexes formed between milk proteins and compounds derived from coffee. β-lactoglobulin, α-lactalbumin, and bovine serum albumin remained for the most part undigested during gastric digestion and were proteolyzed only after the intestinal digestion. On the contrary, caseins (in particular in this study, β-casein was considered) were immediately digested during the gastric phase. The digestibility of HQ milk not heated and of HQ milk and HQ cappuccino after stem injection treatment at 60°C has been evaluated. The degree of hydrolysis, determined by the OPA assay, indicated that there were no significant differences in digestibility between the three samples during the gastric digestion phase. All three samples (HQ milk not heated, HQ milk, and HQ cappuccino both treated at 60°C) underwent greater digestive action during the intestinal phase, but in particular when HQ milk and HQ cappuccino were both treated with steam injection at 60°C. This result clearly indicates that it is the temperature alone that increases the digestibility of the cappuccino and not the presence of compounds in the coffee that can interact with milk proteins. This result on the digestibility of milk and cappuccino is very interesting also in consideration of the fact that in the literature there are very few works on this topic.

It is known that, in the cappuccino, milk proteins can interact with some coffee compounds giving rise to complexes that could affect their bioavailability and beneficial effects, but also could affect the milk protein digestibility. It is in this context that the binding affinity between the principal milk proteins (β-lactoglobulin, α-lactalbumin, β-casein, and lactoferrin) and some coffee compounds (caffeine, chlorogenic acid, and trigonelline) has been determined through fluorescence spectroscopy studies.

All the tested milk proteins showed a high affinity for chlorogenic acid, in particular β-lactoglobulin and lactoferrin, while a low affinity was observed for caffeine and trigonelline.

This aspect, considered the important antioxidant effect of chlorogenic acid, could be very interesting because the possible formation of complexes between the milk proteins and the chlorogenic acid could lead, as previously mentioned, to changes in the efficacy and bioavailability of both molecules, and this certainly deserves to be explored with further future studies.

In general, both the binding studies between milk proteins and coffee compounds and the *in vitro* digestion studies can be considered a good starting point for future investigations into the bioavailability and digestibility of these complexes that are formed inside a cappuccino cup, given that in literature, especially on this last aspect of digestibility, there are no supporting scientific works.

7. REFERENCES

Acharya, D.P.; Sanguansri, L.; Augustin, M.A. Binding of resveratrol with sodium caseinate in aqueous solutions. 2013. Food Chemistry, 141, 1050–1054.

Adler-Nissen, J. Enzimatic hydrolysis of food proteins. 1986. Elsevier Applied Science Publishers.

Ajmal, M.; Nadeem, M.; Imran, M.; Junaid, M. Lipid compositional changes and oxidation status of ultra-high temperature treated Milk. 2018. Lipids in Health and Disease, 17, 227.

Akoglu, B.; Faust, D.; Milovic, V.; Stein, J. Folate and chemoprevention of colorectal cancer: Is 5-methyl-tetrahydrofolate an active antiproliferative agent in folate-treated colon-cancer cells? 2001. Nutrition, 17, 652-3.

AL Doghaither, H.A.; Alyousef, M.M.; AL Selami, N.M.; Al-Ghafari, A.B.; Omar, U.M. Addition of evaporated milk alters antioxdant properties of most commonly consumed coffee in Saudi Arabia. 2017. Malaysian Applied Biology Journal, 46, 97–104.

Albalá-Hurtado, S.; Novella-Rodríguez, S.; Veciana-Nogués, M.T.; Mariné-Font, A. Determination of vitamins A and E in infant milk formulae by high-performance liquid chromatography. 1997. Journal of Chromatography A, 778, 243-246.

Albalá-Hurtado, S.; Veciana-Nogués, T.; Izquierdo-Pulido, M.; Mariné-Font, A. Determination of water-soluble vitamins in infant milk by high performance liquid chromatography. 1997. Journal of Chromatography A, 778, 247–253.

Alongi, M.; Calligaris, S.; Anese, M. Fat concentration and high-pressure homogenization affect chlorogenic acid bioaccessibility and a-glucosidase inhibitory capacity of milk-based coffee beverages. 2019. Journal of Functional Foods, 58, 130–137.

Andersson, I.; Öste, R. Nutritional quality of pasteurized milk. Vitamin B12, folate and ascorbic acid content during storage. 1994. International Dairy Journal, 4, 161-172.

Anema, S.G. The whey proteins in milk: Thermal denaturation, physical interactions, and effects on the functional properties of milk. 2020. Milk proteins (3rd edition), 325-384.

Anema, S.G.; Li, Y. Effect of pH on the association of denatured whey proteins with casein micelles in heated reconstituted skim milk. 2003b. Journal of Agricultural and Food Chemistry, 51, 1640–1646.

Augustin, M.A. Mineral salts and their effect on milk functionality. 2000. Australian Journal of Dairy Technology, 55, 61-64.

Baer, A.; Oroz, M.; Blanc, B. Serological studies on heat-induced interactions of α-lactalbumin and milk-proteins. 1976. Journal of Dairy Research, 43, 419–432.

Ballarini, G. La Preziosa Schiuma del Latte. 2022. Ruminantia.

Bandyopadhyay, P.; Ghosh, A.K.; Ghosh, C. Recent developments on polyphenol–protein interactions: effects on tea and coffee taste, antioxidant properties and the digestive system. 2012. Food & Function, 3, 592–605.

Basnet, P.; Matsushige, K.; Hase, K.; Kadota, S.; Namba, T. Four di-O-caffeoyl quinic acid derivatives from propolis. Potent hepatoprotective activity in experimental liver injury models. 1996. Biological and Pharmaceutical Bulletin, 19, 1479–1484.

Belay, A. Some biochemical compounds in coffee beans and methods developed for their analysis. 2011. International Journal of Physical Sciences, 9, 6373-6378.

Bhagat, A.R.; Delgado, A.M.; Issaoui, M.; Chammem, N.; Fiorino, M.; Pellerito, A.; Natalello, S. Review of the role of fluid dairy in delivery of polyphenolic compounds in the diet: chocolate milk, coffee beverages, matcha green tea, and beyond. 2019. Journal of AOAC International, 102, 1365–1372.

Bogan, K.L., Brenner, C. Nicotinic acid, nicotinamide, and nicotinamide riboside: a molecular evaluation of NAD+ precursor vitamins in human nutrition. 2008. Annual Review of Nutrition, 28, 115-130.

Boushey, C.J.; Beresford, A.A.; Omen, G.S.; Motulsky, A.G. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. 1996. Journal of the American Medical Association, 274, 1049–1057.

Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. 1976. Analytical Biochemistry, 72, 249–254.

Brancaccio, M.; Mennitti, C.; Cesaro, A.; Fimiani, F.; Vano, M.; Gargiulo, B.; Caiazza, M.; Amodio, F.; Coto, I.; D'Alicandro, G.; et al. The Biological Role of Vitamins in Athletes' Muscle, Heart and Microbiota. 2022. International Journal of Environmental Research and Public Health, 19, 1249.

Brick, T.; Ege, M.; Boeren, S.; Böck, A.; von Mutius, E.; Vervoort, J.; Hettinga, K. Effect of Processing Intensity on Immunologically Active Bovine Milk Serum Proteins. 2017. Nutrients, 9, 963.

Brodkorb, A.; Egger, L.; Alminger, M.; Alvito, P.; Assunção, R.; Ballance, S.; Bohn, T.; Bourlieu-Lacanal, C.; Boutrou, R.; Carrière, F.; Clemente, A.; Corredig, M.; Dupont, D.; Dufour, C.; Edwards, C.; Golding, M.; Karakaya, S.; Kirkhus, B.; Le Feunteun, S.; Lesmes, U.; Macierzanka, A.; Mackie, A.R.; Martins, C.; Marze, S.; McClements, D.J.; Ménard, O.; Minekus, M.; Portmann,R.; Santos, C.N.; Souchon, I.; Singh, R.P.; Vegarud, G.E.; Wickham, M.S.J.; Weitschies, W.; Recio, I. INFOGEST static in vitro simulation of gastrointestinal food digestion. 2019. Nature Protocols, 14, 991–1014.

Broyard, C.; Gaucheron, F. Modifications of structures and functions of caseins: a scientific and technological challenge. 2015. Dairy Science and Technology, 95, 831-862.

Bujacz, A. Structures of bovine, equine and leporine serum albumin. 2012. Acta Crystallographica Section D: Biological Crystallography, 68, 1278-1289.

Burg, A.; Silberstein, T.; Yardeni, G.; Tavor, D.; Blumenfeld, J.; Zilbermann, I.; Saphier O. Role of Radicals in the Lipid Peroxidation Products of Commercial Infant Milk Formula. 2010. Journal of Agricultural and Food Chemistry, 58, 2347–2350.

Cai, H.; Liu, X.; Zou, J.; Xiao, J.; Yuan, B.; Li, F.; Cheng, Q. Multi-wavelength spectrophotometric determination of hydrogen peroxide in water with peroxidase-catalyzed oxidation of ABTS. 2018. Chemosphere, 193, 833-839.

Castle, T. J.; Nielsen, J. The great coffee book. 1999. California, USA: Springer Science & Business.

Cawthern, K.M.; Narayan, M.; Chaudhuri, D.; Permyakov, E.A.; Berliner, L.J. Interactions of α-Lactalbumin with Fatty Acids and Spin Label Analogs. 1997. Journal of Biological Chemistry, 272, 30812–30816.

Chanphai, P.; Bourassa, P.; Kanakis, C.D.; Tarantilis, P.A.; Polissiou, M.G.; Tajmir-Riahi, H.A. Review on the loading efficacy of dietary tea polyphenols with milk proteins. 2018. Food Hydrocolloids, 77, 322–328.

Cheison, S.C.; Leeb, E.; Toro-Sierra, J.; Kulozik, U. Influence of hydrolysis temperature and pH on the selective hydrolysis of whey proteins by trypsin and potential recovery of native alpha-lactalbumin. 2011. International Dairy Journal, 21, 166-171.

Cho, Y.; Singh, H.; Creamer, L.K. Heat-induced interactions of β-lactoglobulin A and κ-casein B in a model system. 2003. Journal of Dairy Research, 70, 61–71.

Choi, J.; Ho, J.; Curry, S.; Qin, D.; Bittman, R.; Hamilton, J.A. Interactions of very long-chain saturated fatty acids with serum albumin. 2002. Journal of Lipid Research, 43, 1000-1010.

Church, F.C.; Porter, D.H.; Catignani, G.L.; Swaisgood, H.E. An o-Phthalaldehyde Spectrophotometric Assay For Proteinases. 1985. Analytical Biochemistry, 146, 343-348.

Clifford, M.N.; Johnston K.L.; Knight, S.; Kuhnert N. Hierarchical scheme for LC-MS n identification of chlorogenic acids. 2003. Journal of Agricultural and Food Chemistry, 51, 2900–2911.

Cornish, J.; Palmano, K.; Callon, K.E.; Watson, M.; Lin, J.M.; Valenti, P.; Naot, D.; Grey, A.B.; Reid, I.R. Lactoferrin and bone; structure-activity relationships. 2006. Biochemistry and Cell Biology, 84, 297–302.

Corredig, M.; Dalgleish, D.G. Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. 1996b. Food Research International, 29, 49–55.

Corredig, M.; Dalgleish, D.G. The binding of α-lactalbumin and β-lactoglobulin to casein micelles in milk treated by different heating systems. 1996a. Milchwissenschaft, 51, 123–127.

Corti, R.; Binggeli, C.; Sudano, I.; Spieker, L.; Hanseler E.; Ruschitzka, F.; Chaplin, W.F.; Luscher, T.F.; Noll, G. Coffee acutely increases sympathetic nerve activity and blood pressure independently of caffeine content: Role of habitual versus nonhabitual drinking. 2002. Circulation, 106, 2935–2940.

Custodio-Mendoza, J.A.; Caamaño-Fernandez, C.; Lage, M.A.; Almeida, P.J.; Lorenzo, R.A.; Carro, A.M. GC–MS determination of malondialdehyde, acrolein, and 4-hydroxy-2-nonenal by ultrasound-assisted dispersive liquid-liquid microextraction in beverages. 2022. Food Chemistry, 384, 132530.

Dai, T.; Chen, J.; McClements, D.J.; Hu, P.; Ye, X.; Liu, C.; Li, T. Protein–polyphenol interactions enhance the antioxidant capacity of phenolics: analysis of rice glutelin–procyanidin dimer interactions. 2019. Food & Function, 10, 765-774.

Dai, T.; McClements, D.J.; Hu, T.; Chen, J.; He, X.; Liu, C.; Sheng, J.; Sun, J. Improving foam performance using colloidal protein-polyphenol complexes: Lactoferrin and tannic acid. 2021. Food Chemistry, 377, 131950.

Dalgleish, D.G.; Corredig, M. The Structure of the Casein Micelle of Milk and Its Changes During Processing. 2012. Food Science and Technology, 3, 449-467.

Dalgleish, D.G.; Horne, D.S. A photon correlation spectroscopy study of size distributions of casein micelle suspensions. 1985. European Biophysics Journal, 11, 249–58.

Dalgleish, D.G.; Horne, D.S.; Law, A.J.R. Size-related differences in bovine casein micelles. 1989. Biochimica et Biophysica Acta, 991, 383–387.

Daly, L.E.; Kirke, P.N.; Molloy, A.; Weir, D.G.; Scott, J.M. Folate levels and neural tube defects. 1995. Journal of the American Medical Association, 247, 1698–702.

Davoodi, S.H.; Shahbazi, R.; Esmaeili, S.; Sohrabvandi, S.; Mortazavian, A.; Jazayeri, S.; Taslimi, A. Health-Related Aspects of Milk Proteins. 2016. Iranian Journal of Pharmceutical Research, 15, 573-591.

de Kruif, C.G. Casein micelle interactions. 1999. International Dairy Journal, 9, 183–188.

de Kruif, C.G. Supra-aggregates of casein micelles as a prelude to coagulation. 1998. Journal of Dairy Science, 81, 3019–3028.

Delacroix-Buchet, A.; Lefier, D.; Nuits-Petit, V. Polymorphisme de la caseine κ de trois races bovines francaises et aptitude a la coagulation. 1993. Lait 7, 61–72.

DeWit, J. N.; Swinkels, G.A. A Differential Scanning Calorimetric Study of the Thermal Denaturation of Bovine b-Lactoglobulin: Thermal Behavior at Temperatures up to 100 °C. 1980. Biochimica et Biophysics Acta, 624, 40-50.

Dolgikh, D.A.; Gilmanshin, R.I.; Brazhnikov, E.V.; Bychkova, V.E.; Sernisotnov, G.V.; Venyarninov, S.Y.; Ptitsyn, O.B. 1981. FEBS Letters, 136, 311-315.

Duarte, G.S.; Farah, A. Effect of simultaneous consumption of milk and coffee on chlorogenic acids' bioavailability in humans. 2011. Journal of Agricultural and Food Chemistry, 59, 7925–7931.

Dupas, C.; Marsset-Baglieri, A.; Ordonaud, C.; Tome, D.; Maillard, M.N. Chlorogenic acid is poorly absorbed, independently of the food matrix: A Caco-2 cells and rat chronic absorption study. 2006a. Molecular Nutrition & Food Research, 50, 1053–1060.

Dupas, C.J.; Marsset-Baglieri, A.C.; Ordonaud, C.S.; Ducept, F.M.G.; Maillard, M.N. Coffee Antioxidant Properties: Effects of Milk Addition and Processing Conditions. 2006. Journal of Food Science, 71, 253-258.

Dupont, M. Comparison of the Thermodenaturation of b-Lactoglobulins A and B at pH 6.85. 1965a. Biochimica et Biophysics Acta, 94, 573-575.

Dupont, M. Study of a Reversible Step in the Thermodenaturation of Bovine b-Lactoglobulin A. 1965b. Biochimica et Biophysics Acta, 102, 500-513.

Elfagm, A.A.; Wheelock, J.V. Heat interaction between α-lactalbumin, β-lactoglobulin and casein in bovine milk. 1978. Journal of Dairy Science, 61, 159–163.

El-Messery, T. M.; Mwafy, E.A.; Mostafa, A.M.; El-Din, H.M.F; Mwafy, A.; Amarowicz, R.; Ozcelik, B. Spectroscopic studies of the interaction between isolated polyphenols from coffee and the milk proteins. 2020. Surfaces and Interfaces, 20, 100558.

Farah, A. Coffee as a speciality and functional beverage. 2009. In Functional and speciality beverage technology, 370–395.

Farrell, H.M. Jr.; Jimenez-Flores, R.; Black, G.T.; Butler, J.E.; Creamer, L.K. 2004. Nomenclature of the proteins of cow's milk-6th Revision. 2004. Journal of Dairy Science, 87, 1641–1674.

Farrell, H.M. Jr; Malin, E.L.; Brown, E.M.; Qi, P.X. Casein micelle structure: What can be learned from milk synthesis and structural biology? 2006. Current Opinion in Colloid & Interface Science, 11, 135–47.

Fersht, A. Enzyme Structure and Mechanisms. 1977. Freeman: San Francisco, 18, 321.

Forman, J.P.; Rimm, E.B.; Stampfer, M.J.; Curhan, G.C. Folate intake and the risk of incident hypertension among US women. 2005. Journal of the American Medical Association, 293, 320-329.

Forssen, K.M.; Jagerstad, M.I.; Wigertz, K.; Witthoft, C.M. Folates and dairy products: a critical update. 2000. Journal of the American College of Nutrition, 19, 100S-110S.

Fox, P.F.; Brodkorb, A. The casein micelle: historical aspects, current concepts and significance. 2008. International Dairy Journal, 18, 677–684.

Fried, E. The lowdown on caffe latte. 1993. Black Enterprise 24, 139.

Fu, J.; Savage, P.E.; LuInd, X. Hydrothermal Decarboxylation of Pentafluorobenzoic Acid and Quinolinic Acid. 2009. Industrial & Engineering Chemistry Research, 48, 10467-10471.

Gai, N.; Uniacke-Lowe, T.; O'Regan, J.; Faulkner, H.; Kelly, A.L. Effect of Protein Genotypes on Physicochemical Properties and Protein Functionality of Bovine Milk: A Review. 2021. Foods, 10, 2409.

Ganji, V.; Kafai, M.R. Frequent consumption of milk, yogurt, cold breakfast cereals, peppers, and cruciferous vegetables and intakes of dietary folate and riboflavin but not vitamins B-12 and B-6 are inversely associated with serum total homocysteine concentrations in the US population. 2004. The American Journal of Clinical Nutrition, 80, 1500-7.

Ghayour, N.; Hosseini, S.M.H.; Eskandari, M.H.; Esteghlal, S.; Nekoei, A.-R.; Gahruie, H.H.; et al.; Naghibalhossaini, F. Nanoencapsulation of quercetin and curcumin in casein-based delivery systems. 2018. Food Hydrocolloids, 87, 394-403.

Giansanti, F.; Panella, G.; Leboffe, L.; Antonini, G.; Lactoferrin from Milk: Nutraceutical and Pharmacological Properties. 2016. Pharmaceuticals (Basel), 9, 61.

Goh, J.; Kravchuk, O.; Deeth, H.C. Comparison of mechanical agitation, steam injection and air bubbling for foaming milk of different types. 2009. Milchwissenschaft, 64, 121-124.

Graulet, B.; Girard, C.L.B. Vitamins in Cow Milk: Their Relevance to Human Health. 2017. In Dairy in Human Health and Disease across the Lifespan; Watson, R.R., Collier, R.J., Preedy, V.R., Eds.; Academic Press: Cambridge, MA, USA, Chapter 15, 211–224.

Hakansson, A.; Svensson, M.; Mossberg, A.K.; Sabharwal, H.; Linse, S.; Lazou, I.; Lonnerdal, B.; Svanborg, C. A folding variant of alpha-lactalbumin with bactericidal activity against Streptococcus pneumoniae. 2000. Molecular Microbiology, 35, 589–600.

Hakansson, A.; Zhivotovsky, B.; Orrenius, S.; Sabharwal, H.; Svanborg, C. Apoptosis induced by a human milk protein. 1995. Proceedings of the National Academy of Sciences of the United States of America, 92, 8064–8068.

Haque, Z.; Kinsella, J.E. Interaction between heated κ-Casein and β-lactoglobulin: predominance of hydrophobic interactions in the initial stages of complex formation. 1988. Journal of Dairy Research, 55, 67–80.

Haque, Z.; Kristjansson, M.M.; Kinsella, J.E. Interaction between κ-casein and β-lactoglobulin: possible mechanism. 1987. Journal of Agricultural and Food Chemistry, 35, 644–649.

Haug, A.; Høstmark, A.T.; Harstad, O. Bovine milk in human nutrition – a review. 2005. Lipids in Health ad Desease, 6, 1-16.

Hayes, K.; Pronczuk, A.; Perlman, D. Vitamin E in fortified cow milk uniquely enriches human plasma lipoproteins. 2001. The American Journal of Clinical Nutrition, 74, 211-218.

He, Z.; Chen, J.; Moser, S.E.; Jones, O.G.; Ferruzzi, M.G. Interaction of β-casein with (−)-epigallocatechin-3-gallate assayed by fluorescence quenching: effect of thermal processing temperature. 2015. International Journal of Food Science & Technology, 51, 342–348.

He, Z.; Xu, M.; Zeng, M.; Qin, F.; Chen, J. Interactions of milk α- and β-casein with malvidin-3-O-glucoside and their effects on the stability of grape skin anthocyanin extracts. 2016. Food Chemistry, 199, 314–322.

Hidden, F.; Boomsma, J.; Schins, A.; van den Berg, E. Cappuccino and Specific Heat Versus Heat of Vaporization. 2012. American Association of Physics Teacher, 50, 103–104.

Hiramoto, K.; Kida, T.; Kikugawa, K. Increased urinary hydrogen peroxide levels caused by coffee drinking. 2002. Biological and Pharmceutical Bulletin, 25, 1467-1471.

Hiraoka, Y.; Segawa, T.; Kuwajima, K.; Sugai, S.; Mural, N. α-Lactalbumin: A calcium metalloprotein. 1980. Biochemical and Biophysical Research Communications, 95, 1098-1104.

Ho, T. M.; Bhandari, B.R.; Bansal, N. Functionality of bovine milk proteins and other factors in foaming properties of milk: a review. 2021. Critical Reviews in Food Science and Nutrition, 1–21.

Ho, T.M.; Le, T.H.; Yan, A.; Bhandari, B.R.; Bansal, N. Foaming properties and foam structure of milk during storage. 2019. Food Research International, 116, 379-386.

Holt, C. Casein micelle substructure and calcium phosphate interactions studied by Sephacryl column chromatography. 1998. Journal of Dairy Science, 81, 2994–3003.

Horne, D.S. Casein micelle structure: models and muddles. 2006. Current Opinion in Colloid & Interface Science, 11, 148–153.

Huang, B.X.; Kim, H.; Dass, C.; Probing three-dimensional structure of bovine serum albumin by chemical cross-linking and mass spectrometry. 2004. Journal of the American Society for Mass Spectrometry, 15, 1237–1247.

Huppertz, T. Foaming properties of milk: A review of the influence of composition and processing. 2010. International Journal of Dairy Technology, 63, 477-488.

Hur, S.J.; Lim, B.O.; Decker, E.A.; McClements, D.J. *In vitro* human digestion models for food applications. 2011. Food Chemistry, 125, 1-12.

Indyk, H.E.; Filonzi, E.L.; Gapper, L.W. Determination of minor proteins of bovine milk and colostrum by optical biosensor analysis. 2006. The Journal of AOAC INTERNATIONAL, 89, 898-902.

INRAN (Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione). 2006. Libro Bianco Sul Latte e i Prodotti Lattiero Caseari: Analisi Delle Conoscenze Scientifiche e Considerazioni Sul Valore Del Consumo di Latte e Derivati; Assolatte, M., Ed.; Assolatte: Milano, Italy, Chapter 3, 280–283.

Insel P.; Turner, R.E.; Ross, D. Nutrition. 2004. American dietetic association, Jones and Bartlett, USA, Second Edition.

Ipsen, R.; Otte, J. The relation between protein structure, interfacial rheology and foam formation for various milk proteins. 2004. Annual Transaction of the Nordic Rheology Society, Vol 12.

Jakobek, L. Interactions of polyphenols with carbohydrates, lipids and proteins. 2015. Food Chemistry, 175, 556–567.

Jenssen, H.; Ling, J.R., Ed. Antimicrobial activity of lactoferrin and lactoferrin derived peptides. In Dietary Protein Research Trends. 2007. Nova Science Publisher Inc.: New York, NY, USA, 1–62.

Jeon, J.S.; Kim, H.T.; Jeong, I.H.; Hong, S.R.; Oh M.S.; Yoon, M.H.; Shim, J.H.; Jeong, J.H.; El-Aty, A.A. Contents of chlorogenic acids and caffeine in various coffee-related products. 2019. Journal of Advanced Research, 17, 85–94.

Jeurnink, T.J.M.; de Kruif, C.G. Changes in milk on heating: viscosity measurements. 1993. Journal of Dairy Research, 60, 139–150.

Jiang, J.; Zhang, Z.; Zhao, J.; Liu, Y. The effect of non-covalent interaction of chlorogenic acid with whey protein and casein on physicochemical and radical-scavenging activity of in vitro protein digests. 2018. Food Chemistry, 268, 334–341.

Jimenez-Junca, C.; Sher, A.; Gumy, J.C.; Niranjan, K. Production of milk foams by steam injection: The effects of steam pressure and nozzle design. 2015. Journal of Food Engineering, 166, 247–254.

Jovic, T.H.; Ali, S.R.; Ibrahim, N.; Jessop, Z.M.; Tarassoli, S.P.; Dobbs, T.D.; Holford, P.; Thornton, C.A.; Whitaker, I.S. Could Vitamins Help in the Fight Against COVID-19? 2020. Nutrients, 12, 2550.

Kamath, S.; Huppertz, T.; Houlihan, A.V.; Deeth H.C. The influence of temperature on the foaming of milk. 2008. International Dairy Journal, 18, 994–1002.

Kamiloglu, S.; Van Camp, J.; Capanoglu, E. Black carrot polyphenols: effect of processing, storage and digestion - an overview. 2018. Phytochemistry Reviews, 17, 379-395.

Kapusta, A.; Kuczyńska, B.; Puppel, K. Relationship between the degree of antioxidant protection and the level of malondialdehyde in high-performance Polish Holstein-Friesian cows in peak of lactation. 2018. PLoS One, 13, 0193512.

Kaur, J.; Katopo, L.; Hung, A.; Ashton, J.; Kasapis, S. Combined spectroscopic, molecular docking and quantum mechanics study of β -casein and p -coumaric acid interactions following thermal treatment. 2018. Food Chemistry, 252, 163–170.

Kaushik, S.; Wander, R.; Leonard, S.; German, B.; Traber, M.G. Removal of fat from Cow's milk decreases the vitamin E contents of the resulting dairy products. 2001. Lipids, 36, 73-78.

Kella, N.K.D.; Kinsella, J.E. Enhanced Thermodynamic Stability of b-Lactoglobulin at Low pH: A Possible Mechanism. 1988. Biochemical Journal, 255, 113-118.

Kenneally, P. Hey hipsters, hands off my flat white. 2014. The Guardian. Retrieved September, 20.

Kim, L-J.; Chalmers, T.J.; Smith, G. C.; Das, A.; Keung Poon, E.W.; Wang, J.; Tucker, S. P.; Sinclair, D.A.; Quek, L-E.; Wu, L.E. Nicotinamide mononucleotide (NMN) deamidation by the gut microbiome and evidence for indirect upregulation of the NAD+ metabolome. 2020.

Kinsella, J.E. Functional properties of proteins: Possible relationships between structure and function in foams. 1981. Food Chemistry, 7, 273-288.

Kinsella, J.E.; Melachouris, N. Functional properties of proteins in foods: A survey. 1976. Critical Reviews in Food Science and Nutrition, 7, 219-280.

Kinsella, J.E.; Morr, C.V. Milk proteins: Physicochemical and functional properties. 1984. Critical Reviews in Food Science and Nutrition, 21, 197-262.

Klag, M. J., Wang N.Y.; Meoni L.A.; Brancati, F.L.; Cooper, L.A.; Liang, K.Y.; Young, J.H.; Ford, D.E. Coffee intake and risk of hypertension: The Johns Hopkins precursors study. 2002. Archives of Internal Medicine, 162, 657–662.

Köhler, C.; Hakansson, A.; Svanborg, C.; Orrenius, S.; Zhivotovsky, B. Protease activation in apoptosis induced by MAL. 1999. Experimental Cell Research, 249, 260–268.

Kontopidis, G.; Holt, C.; Sawyer, L. Invited review: Beta-lactoglobulin: Binding properties, structure, and function. 2004. Journal of Dairy Science, 87, 785–796.

Krishna, T.C.; Najda, A.; Bains, A.; Tosif, M.M.; Papli ´ nski, R.; Kapłan, M.; Chawla, P. Influence of Ultra-Heat Treatment on Properties of Milk Proteins. 2021. Polymers, 13, 3164.

Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. 1970. Nature, 227, 680–685.

Laursen, I.; Briand, P.; Lykkesfeldt, A.E. Serum albumin as a modulator on growth of the human breast cancer cell line, MCF-7. 1990. Anticancer Research, 10, 343-351.

Lee, S.Y; Morr, C.V.; Ha, E.Y.W. Structural and Functional Properties of Caseinate and Whey Protein Isolate as Affected by Temperature and pH. 1992. Food Science, 57, 1210-1229.

Li, C.H.; Dai, T.T.; Chen, J.; Li, X.; Li, T.; Liu, C.M.; McClements, D.J. Proteinpolyphenol functional ingredients: The foaming properties of lactoferrin are enhanced by forming complexes with procyanidin. 2021. Food Chemistry, 339, 128145.

Li, T.; Li, X.; Dai, T.; Hu, P.; Niu, X.; Liu, C.; Chen, J. Binding mechanism and antioxidant capacity of selected phenolic acid b-casein complexes. 2020. Food Research International, 129, 108802.

Liang, L.; Subirade, M. Beta-Lactoglobulin/Folic Acid Complexes: Formation, Characterization, and Biological Implication. 2010. Journal of Physical Chemistry B, 114, 6707–6712.

Little, E.M.; Holt, C. An equilibrium thermodynamic model of the sequestration of calcium phosphate by casein phosphopeptides. 2004. European Biophysics Journal, 33, 435–447.

Liu, J.; Wang, Q.; Zhang, H.; Yu, D.; Jin, S.; Ren, F. Interaction of chlorogenic acid with milk proteins analyzed by spectroscopic and modeling methods. 2016. Spectroscopy Letters 49, 44–50.

Long, J.E.; Gould, I.A.; van Winkle, Q. Heat-Induced interaction between crude κ-casein and β-lactoglobulin. 1963. Journal of Dairy Science, 46, 1329–1334.

Lorient, D.; Closs, B.; Courthaudon, J.L. Surface properties of the bovine casein components: relationships between structure and foaming properties. 1989. Journal of Dairy Research, 56, 495-502.

Mandrioli, M.; Boselli, E.; Fiori, F.; Rodriguez-Estrada, M.T. Vitamin D3 in High-Quality Cow Milk: An Italian Case Study. 2020. Foods, 9, 548.

Marchin, S.; Putaux, J-L.; Pignon, F.; Leonil, J. Effects of the environmental factors on the casein micelle structure studied by cryo transmission electron microscopy and small angle X-ray scattering/ultrasmall angle X-ray scattering. 2007. Journal of Chemical Physics, 126, 045101.

Marinova, K.G.; Basheva, E.S.; Nenova, B.; Temelska, M.; Mirarefi, A.Y.; Campbell, B.; Ivanov, I.B. Physico-chemical factors controlling the foamability and foam stability of milk proteins: Sodium caseinate and whey protein concentrates. 2009. Food Hydrocolloids, 23, 1864-1876.

Martin, A.H.; Grolle, K.; Bos, M.A.; Stuart, M.A.; van Vliet, T. Network forming properties of various proteins adsorbed at the air/water interface in relation to foam stability. 2002. Journal of Colloid and Interface Science, 254, 175–183.

Mason, J.B. Folate status: effects on carcinogenesis. 1995. In Baily LB (ed): "Folate in Health and Disease." New York: Marcel Dekker, 316–378.

McKenzie, H.A. β-Lactoglobulins. Chemistry and molecular biology. 1971. Milk Proteins, ed. McKenzie, H.A., Academic Press, New York, 257–330.

Hernandez, M.M.J.; Camanas, V.R.M.; Alvarez-Coque, G.M.C. Determination of total free amino acids with o-phthalaldehyde and N-acetyl-L-cysteine. 1990b. Microchemical Journal, 42, 288.

Hernandez, M.M.J.; Camanas, V.R.M.; Cuenca, M.E.; Alvarez-Coque, G.M.C. Determination of the protein and free amino acid content in a sample using o-phthalaldehyde and N-acetyl-L-cysteine. 1990a. Analyst, 115, 1125.

Miller, E.W. The food lover's guide to florence: With culinary excursions in tuscany. 2003. London, UK: Springer Science & Business.

Miranda, G.; Pelissier, J. P. Kinetic Studies of in Vivo Digestion of Bovine Unheated Skim-Milk Proteins in the Rat Stomach. 1983. Journal of Dairy Research, 504, 27-36.

Moeiniafshari, A.-A.; Zarrabi, A.; Bordbar, A.-K. Exploring the interaction of naringenin with bovine beta-casein nanoparticles using spectroscopy. 2015. Food Hydrocolloids, 51, 1–6.

Moreno, F.J.; Mellon, F.A.; Wickham, M.S.J.; Bottrill, A.R.; Mills, E.N.C. Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physiologically relevant in vitro gastrointestinal digestion. 2005. FEBS Journal, 272, 341-352.

Mossberg, A.K.; Hun Mok, K.; Morozova-Roche, L.A.; Svanborg, C. Structure and function of human α-lactalbumin made lethal to tumor cells (HAMLET)-type complexes. 2010. Federation of European Biochemical Societies, 277, 4614–4625.

Muralidhara, B.; Prakash, V. Interaction of 3'-O-caffeoyl D-quinic acid with human serum albumin. 1995. International Journal of Peptide and Protein Research, 46, 1–8.

Ng-Kwai-Hang, K.F.; Kim, S. Different amounts of β-lactoglobulin A and B in milk from heterozygous AB cows. 1996. International Dairy Journal, 6, 689-695.

Nguyen, N.H.A.; Streicher, C.; Anema, S.G. The effect of thiol reagents on the denaturation of the whey protein in milk and whey protein concentrate solutions. 2018. International Dairy Journal, 85, 285–293.

Nguyen, N.H.A.; Wong, M.; Havea, P.; Guyomarc'h, F.; Anema, S.G. The protein interactions and rheological properties of skim milk heated in the presence of low levels of reducing agent. 2013. Food Chemistry, 138, 1604–1609.

Nicolopoulos, K.; Mulugeta, A.; Zhou, A.; Hypponen, E. Association between habitual coffee consumption and multiple disease outcomes: A Mendelian randomisation phenome-wide association study in the UK Biobank. 2020. Clinical Nutrition, 39, 3467–3476.

Niero, G.; Penasa, M.; Berard, J.; Kreuzer, M.; Cassandro, M.; De Marchi, M. Technical note: Development and validation of an HPLC method for the quantification of tocopherols in different types of commercial cow milk. 2018. Journal of Dairy Science, 101, 6866-6871.

Niseteo, T.; Komes, D.; Belscak-Cvitanovic, A.; Horzic, D.; Budec, M. Bioactive composition and antioxidant potential of different commonly consumed coffee brews affected by their preparation technique and milk addition. 2012. Food Chemistry 134, 1870–1877.

Nuhu, A.A. Bioactive Micronutrients in Coffee: Recent Analytical Approaches for Characterization and Quantification. 2014. ISRN Nutrition, 1-13.

Oldfield, D.J.; Singh, H.; Taylor, M.W. Association of β-lactoglobulin and α-lactalbumin with the casein micelles in skim milk heated in an ultra-high temperature plant. 1998b. International Dairy Journal, 8, 765–770.

Oldfield, D.J.; Singh, H.; Taylor, M.W. Kinetics of heat-induced whey protein denaturation and aggregation in skim milks with adjusted whey protein concentration. 2005. Journal of Dairy Research, 72, 369–378.

Oldfield, D.J.; Singh, H.; Taylor, M.W.; Pearce, K.N. Heat-induced interactions of β-lactoglobulin and α-lactalbumin with the casein micelle in pH-adjusted skim milk. 2000. International Dairy Journal, 10, 509–518.

Ortega, N.; Reguant, J.; Romero, M.P.; Macia, A.; Motilva, M.J. Effect of fat content on the digestibility and bioaccessibility of cocoa polyphenol by an in vitro digestion model. 2009. Journal of Agricultural and Food Chemistry, 57, 5743–5749.

Østdal, H.; Bjerrum, M.J.; Pedersen, J.A.; Andersen, H.J. Lactoperoxidase-Induced Protein Oxidation in Milk. 2000. Journal of Agricultural and Food Chemistry, 48, 3939-3944.

Otemuyiwa, I.O.; Williams, M.F.; Adewusi, S.A. Antioxidant activity of health tea infusions and effect of sugar and milk on in-vitro availability of phenolics in tea, coffee and cocoa drinks. 2017. Nutrition & Food Science, 47, 458–468.

Ozdal, T.; Capanoglu, E.; Altay, F. A review on protein–phenolic interactions and associated changes. 2013. Food Research International, 51, 954–970.

Ozyurt, V.H.; Otles, S. Gidalarin yapisindaki fenolik bile- ¸iklerin ve proteinlerin interaksiyon mekanizmalari ve interaksiyona s etki eden faktorler. 2016. GIDA/The Journal of Food.

Parker, F.; Migliore-Samour, D.; Floch, F.; Zerial, A.; Werner, G.H.; Jollès, J.; Casaretto, M.; Zahn, H.; Jollès, P. Immunostimulating hexapeptide from human casein: Amino acid sequence, synthesis and biological properties. 1984. Europena Journal of Biochemistry, 145, 677–682.

Pelligrini, A.; Thomas, U.; Bramaz, N.; Hunziker, P.; von Fellenberg, R. Isolation and identification of three bactericidal domains in the bovine alpha-lactalbumin molecule. 1999. Biochimica et Biophysica Acta, 1426, 439–448.

Pereira, P. Milk nutritional composition and its role in human health. 2014. Nutrition, 30, 619-627.

Permyakov, E.A. α-Lactalbumin, Amazing Calcium-Binding Protein. 2020. Biomolecules, 10(9), 1210.

Permyakov, E.A.; Morozova, L.A.; Burstein, E.A. Cation binding effects on the pH, thermal and urea denaturation transitions in α-lactalbumin. 1985. Biophysical Chemistry, 21, 21-31.

Permyakov, E.A.; Yarmolenko, V.V.; Kalinichenko, L.P.; Morozova, L.A.; Burstein, E.A. α-Lactalbumin binds magnesium ions: Study by means of intrinsic fluorescence technique. 1981b. Bioehemical and Biophysical Research Communications, 100, 191-197.

Permyakova, E.A.; Berliner, L.J. α-Lactalbumin: Structure and function. 2000. Federation of European Biochemical Societies, 473, 269–274.

Perrnyakov, E.A.; Kalinichenko, L.P.; Morozova, L.A.; Yarrnolenko, V.V.; Burstein, E.A. α-Lactalbumin binds magnesium ions: Study by means of intrinsic fluorescence technique. 1981a. Biochemical and Biophysical Research Communications, 102, 1-7.

Picciano, M.F.; West, S.G.; Ruch, A.L.; Kris-Etherton, P.M.; Zhao, G.; Johnston, K.E.; Maddox, D.H.; Fishell, V.K.; Dirienzo, D.B.; Tamura, T. Effect of cow's milk on food folate bioavailability in young women. 2004. The American Journal of Clinical Nutrition, 80, 1565-9.

Pihlanto-Leppala, A.; Rokka, T.; Korhonen, H. Angiotensin I converting enzyme inhibitory peptides from bovine milk proteins. 1998. International Dairy Journal, 8, 325–331.

Poole, R.; Ewings, S.; Parkes, J.; Fallowfield, J.A.; Roderick, P. Misclassification of coffee consumption data and the development of a standardised coffee unit measure. 2019. BMJ Nutrition, Prevention & Health 2, 11–000013.

Poole, R.; Kennedy, O.J.; Roderick, P.; Fallowfield, J.A.; Hayes, P.C.; Parkes, J. 2017. Coffee consumption and health: Umbrella review of meta-analyses of multiple health outcomes. 2017. BMJ, 359, 1–18.

Poulsen, N.A.; Rybicka, I.; Poulsen, H.D.; Larsen, L.B.; Andersen, K.K.; Larsen, M.K. Seasonal variation in content of riboflavin and major minerals in bulk milk from three Danish dairies. 2015. International Dairy Journal, 42, 6–11.

Pruitt, K.M.; Kamau, D.N. Quantitative analysis of bovine lactoperoxidase system components and of the effects of the activated system on bacterial growth and survival. In: Indigenous Antimicrobial Agents of Milk. 1994. International Dairy Federation Brussels, Belgium, 73-87.

Qi, P.X.; Wickham, E.D.; Piotrowski, E.G.; Fagerquist, C.K.; Farrell, H.M. Implication of C-Terminal Deletion on the Structure and Stability of Bovine β-casein. 2005. The Protein Journal, 24, 431–444.

Qian, S.; Sun, J.; Cao, D.; Tuo, Y.; Jiang, S.; Mu, G. Experimental and Modelling Study of the Denaturation of Milk Protein by Heat Treatment. 2017. Korean Journal for Food Science of Animal Resources, 37, 44-51.

Quan, W.; Qie, X.; Chen, Y.; Zeng, M.; Qin, F.; Chen, J.; He, Z. Effect of milk addition and processing on the antioxidant capacity and phenolic bioaccessibility of coffee by using an in vitro gastrointestinal digestion model. 2020. Food Chemistry, 308, 125598.

Raikos, V. Effect of heat treatment on milk protein functionality at emulsion interfaces. A review. 2010. Food Hydrocolloids, 24, 259-265.

Rashidinejad, A.; Birch, E.J.; Hindmarsh, J.; Everett, D.W. Molecular interactions between green tea catechins and cheese fat studied by solid-state nuclear magnetic resonance spectroscopy. 2017. Food Chemistry, 215, 228–234.

Rosa, L.; Cutone, A.; Lepanto, M.S.; Paesano, R.; Valenti, P. Lactoferrin: A Natural Glycoprotein Involved in Iron and Inflammatory Homeostasis. 2017. International Journal of Molecular Sciences, 18, 1985.

Rouge, P.; Cornu, A.; Biesse-Martin, A.S.; Lyan, B.; Rochut, N.; Graulet, B. Identification of quinoline, carboline and glycinamide compounds in cow milk using HRMS and NMR. 2013. Food Chemistry, 141, 1888-1894.

Ruegg, M.; Moor, U.; Blanc, B. A calorimetric study of thermal denaturation of whey proteins in simulated milk ultrafiltrate. 1977. Journal of Dairy Research, 44, 509–520.

Rutten, M.J.M.; Bouwman, A.C.; Sprong, R.C.; van Arendonk, J.A.M.; Viske, M.H.P.W. Genetic Variation in Vitamin B-12 Content of Bovine Milk and Its Association with SNP along the Bovine Genome. 2013. PLoS one, 8, 62382.

Sadiq, U.; Gill, H.; Chandrapala, J. Casein Micelles as an Emerging Delivery System for Bioactive Food Components. 2021. Foods, 10, 1965.

Sánchez-González, I.; Jiménez-Escrig, A.; Saura-Calixto F. In vitro antioxidant activity of coffees brewed using different procedures (Italian, espresso and filter). 2005. Food Chemistry, 90, 133–9.

Sarkar, A.; Singh, H. Emulsions and Foams Stabilised by Milk Proteins. 2015. McSweeney, P.L.H.; O'Mahony, J.A. (eds.) Advanced Dairy Chemistry 1B Proteins: Applied Aspects, 133-153.

Sawyer, W.H. Complex between β-lactoglobulin and κ-casein. A review. 1969. Journal of Dairy Science, 52, 1347–1355.

Sawyer, W.H.; Coulter, S.T.; Jenness, R. Role of sulfhydryl groups in the interaction of κ-casein and β-lactoglobulin. 1963. Journal of Dairy Science, 46, 564–565.

Schmelzer, C.E.H.; Schöps, R.; Reynell, L.; Ulbrich-Hofmann, R.; Neubert, R.H.H.; Raith, K. Peptic digestion of β-casein. 2007. Journal of Chromatography A, 1166, 108–115.

Schmidt, A.; Pratsch, H.; Schreiner, M.G.; Mayer, H.K. Determination of the native forms of vitamin B1 in bovine milk using a fast and simplified UHPLC method. Food Chemistry, 229, 452–457.

Schmidt, D.G. Association of caseins and casein micelle structure. 1982. Developments in Dairy Chemistry, ed. PF Fox, 1, 61–86.

Schramm, D.D.; Karim, M.; Schrader, H.R.; Holt, R.R.; Kirkpatrick, N.J.; Polagruto, J.A.; Ensunsa, J.L.; Schmitz, H.H.; Keen, C.L. Food effects on the absorption and pharmacokinetics of cocoa flavanols. 2003. Life Sciences, 73, 857–869.

Scudiero, O.; Lombardo, B.; Brancaccio, M.; Mennitti, C.; Cesaro, A.; Fimiani, F.; Gentile, L.; Moscarella, E.; Amodio, F.; Ranieri, A.; et al. Exercise, immune system, nutrition, respiratory and cardiovascular diseases during COVID-19: A complex combination. 2021. International Journal of Environmental Research and Public Health, 18, 904.

Sharma, S.; Singh, A.K.; Kaushik, S.; Sinha, M.; Singh, R.P.; Sharma, P.; Sirohi, H.; Kaur, P.; Singh, T.P. Lactoperoxidase: structural insights into the function,ligand binding and inhibition. 2013. International Journal of Biochemistry and Molecular Biology, 4, 108-28.

Shevchenko, A.; Tomas, H.; Havliš, J.; Olsen, J.V.; Mann, M. In gel digestion for mass spectrometric characterization of proteins and proteomes. 2006. Nature Protocols, 1, 2856-2860.

Shin, K.; Wakabayashi, H.; Yamauchi, K.; Teraguchi, S.; Tamura, Y.; Kurokawa, M.; Shiraki, K. Effects of orally administered bovine lactoferrin and lactoperoxidase on influenza virus infection in mice. 2005. Journal of Medical Microbiology, 54, 717–723.

Siebert, K.J.; Troukhanova, N.V.; Lynn, P.Y. Nature of polyphenol-protein interaction. 1996. Journal of Agricultural and Food Chemistry, 44, 80–85.

Silva, S.V.; Malcata, F.X. Caseins as a source of bioactive peptides. 2005. International Dairy Journal, 15, 1–15.

Singh, H.; Creamer, L.K. Denaturation, aggregation and heat-stability of milk protein during the manufacture of skim milk powder. 1991a. Journal of Dairy Research, 58, 269–283.

Singh, H.; Ye, A. Interactions and functionality of milk proteins in food emulsions. 2020. Milk Proteins (3rd edition), 467-497.

Sinha, M.; Kaushik, S.; Kaur, P.; Sharma, S.; Singh, T.P. Antimicrobial Lactoferrin Peptides: The Hidden Players in the Protective Function of a Multifunctional Protein. 2013. International Journal of Peptide Research and Therapeutics. 2013, 390230.

Smith, S.G.; Lewis, M.; Aschaffenburg, R. Crystallographic analysis of the three-dimensional structure of baboon α-lactalbumin at low resolution. Homology with lysozyme. 1987. The Biochemical Joural, 242, 353-360.

Smits, P.; van Brouwershaven, J.H. Heat-induced association of β-lactoglobulin and casein micelles. 1980. Journal of Dairy Research, 47, 313–325.

Sanchez-Gonzalez, I.; Jimenez-Escrig, A.; Saura-Calixto, F. 2005. In vitro antioxidant activity of coffees brewed using different procedures (Italian, espresso and filter). 2005. Food Chemistry 90, 133–139.

Spellman, D.; McEvoy, E.; O'Cuinn, G.; FitzGerald, R.J. Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. 2003. International Dairy Journal, 13, 447–453.

Staff, A.C.; Holven, K.; Løken, E.B.; Sygnestveit, K.; Vollset, S.E.; Smeland, S. Does folic acid have effects on other health problems than neural tube defects? 2005. Tidsskr Nor Laegeforen, 125, 438-441.

Stojadinovic, M.; Radosavljevic, J.; Ognjenovic, J.; Vesic, J.; Prodic, I.; Stanic-Vucinic, D.; Velickovic, T.C. Binding affinity between dietary polyphenols and b-lactoglobulin negatively correlates with the protein susceptibility to digestion and total antioxidant activity of complexes formed. 2013. Food Chemistry, 136, 1263–1271.

Swaisgood, H.E. Chemistry of the caseins. 2003. Advanced Dairy Chemistry, 1, 139–202.

Tagliazucchi, D.; Helal, A.; Verzelloni, E.; Conte, A. The type and concentration of milk increase the in vitro bioaccessibility of coffee chlorogenic acids. 2012. Journal of Agricultural and Food Chemistry, 60, 11056–11064.

Tanaka, T. Antimicrobial activity of lactoferrin and lactoperoxidase in milk. In Dietary Protein Research Trends. Ling, J.R., Ed. 2007. Nova Science Publisher Inc.: New York, NY, USA, 101–115.

Teller, D.C.; Swanson, E.; DeHaen, C. The Translational Friction Coefficient of Proteins. 1979. Methods in Enzymology, 61, 104-124.

Tenovuo, J.O. The peroxidase system in human secretions. In The Lactoperoxidase System: Chemistry and Biological Significance; Pruitt, K.M., Tenovuo, J.O., Eds. 1985. Marcel Dekker: New York, NY, USA, 101–122.

Timasheff, S.N.; Townend, R.; Mescanti, L.The Optical Rotatory Dispersion of the b-Lactoglobulins. 1966. Journal of Biological Chemistry, 241, 1863-1870.

Tong, L.M.; Sasaki, S.; McClements, D.J.; Decker, E.A. Mechanisms of the Antioxidant Activity of a High Molecular Weight Fraction of Whey. 2000. Journal of Agricultural and Food Chemistry, 48, 1473-1478.

Udabage, P.; McKinnon, I.R.; Augustin, M.A. The use of sedimentation field-flow fractionation and photon correlation spectroscopy in the characterization of casein micelles. 2003. Journal of Dairy Research, 70, 453–459.

Uppu, S.N.; London, B.K. Hydrogen Peroxide Levels in Freshly Brewed Coffee and the Effects of Storage. 2020. BioRxiv, 08, 242552.

USDA, National Nutrient Database for Standard Reference. (accessed September 3, 2007), http://www.nal.usda.gov/fnic/foodcomp/Data/.

Van der Ven, C.; Gruppen, H.; de Bont, D.B.A.; Voragen, A.G.J. Correlations between Biochemical Characteristics and Foam-Forming and -Stabilizing Ability of Whey and Casein Hydrolysates. 2002. Journal of Agricultural and Food Chemistry, 50, 2938–2946.

Vincenzetti, S.; Ariani, A.; Polidori, P. Casein characteristics in equid and human milk. 2016. Nova Science Publisher: New York, NY, USA, 62-66.

Vincenzetti, S.; Polidori, P.; Mariani, P.; Cammertoni, N.; Fantuz, F. et al. Donkey milk protein fractions characterization. 2008. Food Chemistry, 106, 640-649.

Vincenzetti, S.; Polidori, P.; Vita, A. Nutritional characteristics of donkey's milk protein fraction. 2007. In: Dietary Protein Research Trends; Editor: Ling, J.R., Chaper 8, 207-225.

Vincenzetti, S.; Pucciarelli, S.; Polzonetti, V.; Polidori, P. Role of Proteins and of Some Bioactive Peptides on the Nutritional Quality of Donkey Milk and Their Impact on Human Health. 2017. Beverages, 3, 58-77.

Walstra, P. Principles of Foam Formation and Stability. 1989. Springer Series in Applied Biology, 1-15.

Ward, B.R.; Goddard, S.J.; Augustin, M.A.; McKinnon, I.R. EDTA-induced dissociation of casein micelles and its effect on foaming properties of milk. 1997. Journal of Dairy Research, 64, 495–504.

Ward, P.P.; Paz, E.; Conneely, O.M. Multifunctional roles of lactoferrin: A critical overview. 2005. Cellular and Molecular Life Sciences, 62, 2540–2548.

Waugh, D.F.; Creamer, L.K.; Slattery, C.W. Core polymers of casein micelles. 1970. Biochemistry, 9, 786–795.

Weiss, J. N. The Hill Equation Revisited: Uses and Misuses. 1997. The FASEB Journal, 11, 835–841.

Wu, S.; Zhang, Y.; Ren, F.; Qin, Y.; Liu, J.; Liu, J.; et al.; Zhang, H. Structure–affinity relationship of the interaction between phenolic acids and their derivatives and β-lactoglobulin and effect on antioxidant activity. 2018. Food Chemistry, 245, 613–619.

Xiong, X.; Ho, M.T.; Bhandari, B.; Bansal, N. Foaming properties of milk protein dispersions at different protein content and casein to whey protein ratios. 2020. International Dairy Journal, 109, 104758.

Xiong, X.; Ho, M.T.; Bhandari, B.; Bansal, N. Foaming properties of milk protein dispersions at different protein content and casein to whey protein ratios. 2020. International Dairy Journal, 109, 104758.

Yildirim-Elikoglu, S.; Erdem, Y.K. Interactions between milk proteins and polyphenols: Binding mechanisms, related changes, and the future trends in the dairy industry. 2018. Food Reviews International, 34, 665–697.

Yuan, S.; Zhang, Y.; Liu, J.; Zhao, Y.; Tan, L.; Liu, J.; et al.; Zhang, H. Structure-affinity relationship of the binding of phenolic acids and their derivatives to bovine serum albumin. 2019. Food Chemistry, 278, 77–83.

Yvon, M.; Van Hille, I.; Pelissier, J. P.; Guilloteau, P.; Toullec, R. In Vivo Milk Digestion in the Calf Abomasum. II. Milk and Whey Proteolysis. 1984. Reproduction Nutrition Development, 24, 835-843.

Zayas, J.F. Foaming Properties of Proteins. 1997. Functionality of Proteins in Food, 260–309.

Zhang, H.; Yu, D.; Sun, J.; Guo, H.; Ding, Q.; Liu, R.; Ren, F. Interaction of milk whey protein with common phenolic acids. 2014. Journal of Molecular Structure, 1058, 228–233.

Zhang, L.; McClements, D.J.; Wei, Z.L.; Wang, G.Q.; Liu, X.B.; Liu, F.G. Delivery of synergistic polyphenol combinations using biopolymer-based systems: Advances in physicochemical properties, stability and bioavailability. 2019. Critical Reviews in Food Science and Nutrition, 60, 2083–2097.

Zhang, Z.; Dalgleish, D.G.; Goff, H. Effect of pH and ionic strength on competitive protein adsorption to air/water interfaces in aqueous foams made with mixed milk proteins. 2004. Colloids and Surfaces B: Biointerfaces, 34, 113–121.

Zittle, C.A.; Custer, J.H.; Cerbulis, J.; Thompson, M.P. κ-Casein -β-lactoglobulin interaction in solution when heated. 1962. Journal of Dairy Science, 45, 807–810.

8. LIST OF PUBLICATIONS

Publications On Peer-Reviewed Journals and Book Chapter

1. **Proteomic characterization of kefir milk by two-dimensional electrophoresis followed by mass spectrometry**. *Santini, G., Bonazza, F., Pucciarelli, S., Polidori, P., Ricciutelli, M., Klimanova, Y., Silvi, S., Polzonetti, V., Vincenzetti S.* 2020. Journal of Mass Spectrometry. 55, e4635. https://doi.org/10.1002/jms.4635 (Corresponding author: Santini. G.)
2. **B-Vitamins Determination in Donkey Milk**. *Vincenzetti, S., Pucciarelli, S., Santini, G., Klimanova, Y., Polzonetti, V., Polidori, P*. Beverages. 2020. 6, 46. https://doi:10.3390/beverages6030046
3. **Vitamins in Human and Donkey Milk: Functional and Nutritional Role.** *Vincenzetti, S., Santini, G., Polzonetti, V., Pucciarelli, S., Klimanova, Y., Polidori, P*. Nutrients. 2021. 13, 1509. https://doi.org/10.3390/nu13051509
4. **Nutritional Properties of Camelids and Equids Fresh and Fermented Milk.** *Polidori, P., Cammertoni, N., Santini, G., Klimanova, Y., Zhang, JJ., Vincenzetti, S.* Dairy. 2021. 2, 288-302. https://doi.org/10.3390/dairy2020024.
5. **Effects of Donkeys Rearing System on Performance Indices, Carcass, and Meat Quality**. *Polidori, P., Cammertoni, N., Santini, G., Klimanova, Y., Zhang, JJ., Vincenzetti, S.* Foods 2021. 10, 3119. https://doi.org/10.3390/foods10123119
6. **Nutraceutical and Functional Properties of Camelids' Milk.** *Vincenzetti, S., Cammertoni, N., Rapaccetti, R., Santini, G., Klimanova, Y., Zhang, J.J, Polidori, P*. Beverages 2022. 8, 12. https://doi.org/10.3390/beverages8010012
7. **Effects of Ageing on Donkey Meat Chemical Composition, Fatty Acid Profile and Volatile Compounds**. *Polidori, P., Santini, G., Klimanova, Y., Zhang, JJ., Vincenzetti, S.* Foods 2022, 11, 821.https://doi.org/10.3390/foods11060821
8. **Effect of steam frothing on milk microfoam: chemical composition, texture, stability and organoleptic properties**. *Klimanova, Y., Polzonetti, V., Pucciarelli, S., Perinelli, D.R., Bonacucina, G., Cespi, M., Gabrielli, M.G., Santini, G., Fioretti, L., Cognigni, L., Vincenzetti, S.* Submitted to International Dairy Journal, second revision.
9. **Nutritional Parameters in Colostrum of Different Mammalian Species**. Polidori, P., Rapaccetti, R., Klimanova, Y., Zhang, J-J., Santini, G., Vincenzetti, S. Submitted to Beverages, unser review

10. **Biochemical characteristics of cappuccinos made with high-quality pasteurized milk and UHT milk at different steam injection conditions.** *Santini, G., Klimanova, Y., Pucciarelli, S., Polzonetti, V., Cespi, M., Polidori, P., Cognigni, L., Fioretti, L., Vincenzetti, S.* In preparation.

PROCEEDINGS

1. **Milk Characterization for a "high quality" cappuccino**. *Giuseppe Santini, Valeria Polzonetti, Stefania Pucciarelli, Paolo Polidori, Lauro Fioretti,* ***Silvia Vincenzetti***. Cibo e nutraceutici: Parola chiave: Caratterizzazione. 4° Convegno delle Piattaforme Tematiche di Ateneo su "Alimenti e Nutrizione" e "Salute Umana e Animale". Auditorium Benedetto XIII - via Le Mosse - Colle Paradiso, Camerino, 9 luglio 2019.
2. **Proteomic characterization of kefir milk by two-dimensional electrophoresis followed by mass spectrometry**. *Giuseppe Santini, Francesca Bonazza, Stefania Pucciarelli, Paolo Polidori, Massimo Ricciutelli, Yulia Klimanova, Stefania Silvi, Valeria Polzonetti,* ***Silvia Vincenzetti***. "6th Mass Spectrometry Food Day", Camerino 25-27 September, 2019
3. **Vitamins in human, cow, and donkey milk: a comparison of nutritional properties.** ***Silvia Vincenzetti****, Giuseppe Santini, Yulia Klimanova, JingJing Zhang, Natalina Cammertoni, Valeria Polzonetti, Stefania Pucciarelli, Paolo Polidori*. Alimenti e Nutraceutici: salute e prevenzione attraverso il cibo: 5° Convegno a cura delle Piattaforme Tematiche di Ateneo su "Alimenti e Nutrizione" e "Salute Umana e Animale". Evento on-line 13 luglio 2021. ISBN: 978-88-6768-049-8.
4. **Water- and fat-soluble vitamins in milk after different thermal treatments and steam injection**. *Giuseppe Santini, Valeria Polzonetti, Yulia Klimanova, Stefania Pucciarelli, Paolo Polidori, Lauro Fioretti,* ***Silvia Vincenzetti*****.** Alimenti e Nutraceutici: salute e prevenzione attraverso il cibo: 5° Convegno a cura delle Piattaforme Tematiche di Ateneo su "Alimenti e Nutrizione" e "Salute Umana e Animale". Evento on-line 13 luglio 2021. ISBN: 978-88-6768-049-8.
5. **Qualità nutrizionale e "funzionale" del latte e del formaggio biofortificato con tannini micro-incapsulati**. *Silvia Vincenzetti, Giuseppe Santini, Natalina Cammertoni, Paolo Polidori*. Atti del convegno finale del progetto MilkBioActinCaps: Utilizzo di microincapsulati di composti bioattivi da scarti dell'industria alimentare come integratori di mangimi per il miglioramento dell'attitudine fermentativa e della valenza nutraceutica del latte. Editori: Galgano Fernanda e Caruso M. Carmela. Universo Sud Edizioni, 15 ottobre 2021. ISBN: 9788899432737.

PARTICIPATION IN THE MILKBIOACTINCAPS PROJECT

Title of the Project: UTILIZZO DI MICROINCAPSULATI DI COMPOSTI BIOATTIVI DA SCARTI DELL'INDUSTRIA ALIMENTARE COME INTEGRATORI DI MANGIMI PER IL MIGLIORAMENTO DELL'ATTITUDINE FERMENTATIVA E DELLA VALENZA NUTRACEUTICA DEL LATTE

Principal Investigator: Prof. Fernanda Galgano, Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali (SAFE), Università degli Studi della Basilicata

Dr. Santini collaborated with the WP entitled: **Nutritional and "functional" quality of milk and cheese bio-fortified with microencapsulated tannins.**

More information on the project is available at the following link:
www.https://milkbioactincaps.com/