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Protein digestibility of human milk, cow milk and goat milk in the *in vitro* static infant digestion simulation

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ORIGINAL ARTICLE

Abstract

Although the importance of human milk is well known, the search for alternatives continues today. Among these alternatives, goat and cow milk, which are industrial species, are in the first place. The literature has primarily focused on the digestive behaviour of human milk and infant formulas. In this study, the digestive behaviours of human, goat, and cow milks were examined at different stages of an infant digestion simulation. Throughout gastrointestinal digestion, a decrease in particle size was observed in human milk, whereas an increase in particle size was detected in cow and goat milk. The pH values of human milk, cow milk, and goat milk were determined to be 6.66, 6.79, and 6.71, respectively. The dry matter content was reported as 11.75%, 13.62%, and 11.0%. In addition to structural changes, significant increases in antioxidant capacity and ACE inhibitory activity (%) were observed during digestion, particularly in cow and goat milk. These biofunctional developments will shed light on the health benefits of cow and goat milk, as well as their potential use in the development of infant formulas. Characterisation of cow and goat milk digestive behaviour in situations of human milk deficiency will contribute to the development of new infant formulas and alternative products.

Keywords: ace inhibitory activity; antioxidant capacity; cow milk; goat milk; human milk; in vitro digestion; microstructure; particle size

Introduction

Human milk provides an ideal combination of components for infant nutrition, promoting healthy growth through its components and laying the foundation for optimal development (Atyeo and Alter, 2021). With the end of the lactation period and the transition to complementary foods, different types of milk, such as cow, goat, sheep, and buffalo milk, begin to be incorporated into human nutrition (Wang *et al*., 2022; He *et al*., 2022). Due to human dietary habits and higher production quantities, cow milk is considered the primary alternative suitable for industrial production. Goat milk is an alternative milk type that stands out for its unique composition, health-promoting properties, and similarity to human milk. While goat milk is defined as the closest alternative to human milk in cases where human milk is unavailable or insufficient, its availability is limited due to production quantities (Prosser, 2021; Liu *et al*., 2023). Additionally, the increasing number of studies on goat milk and its similarities to human milk

(Altun and Sarıcı, 2017; Clark and Mora Garcia, 2017; Prosser, 2021) has placed goat milk in the second position after cow milk. In recent years, it has been reported that the rate of exclusive breastfeeding during the first six months has decreased to approximately 40% due to various factors (Kartal & Gürsoy, 2020). In light of these data, it is considered essential to gain a more detailed understanding of alternatives in infant nutrition in cases where human milk is unavailable or insufficient. Human milk provides complete infant nutrition with the macro/micronutrients it contains; however, studies have reported that human and cow milk protein compositions differ in terms of digestibility (Huppertz and Chia, 2021). It has been reported that while human milk forms a soft clot in the infant stomach, which transitions into an easily digestible form, cow milk forms a harder clot, making digestion more difficult (Roy *et al*., 2020). This is generally associated with the protein structure of the milk type. There are described differences in protein composition among human, cow, and goat milk, with the most important difference being the protein ratios (casein:whey) in humans, cows, and goats, respectively: 40:60, 80:20, and 80:20. The protein content of goat milk is quite similar to that of cow milk, and while the main casein fractions (CN) are αs1-CN, αs2-CN, β-CN, and k-CN, the whey proteins include β-lactoglobulin, α-lactalbumin, immunoglobulins, and glycomacropeptides (Altun and Sarıcı, 2017; Waang *et al*., 2019).

The better digestibility makes goat milk stand out as a crucial parameter in baby nutrition (Park, 1994; Dhasmana *et al*., 2022). Goat milk has been identified as an important source of taurine for both infant nutrition and adults. Several studies are available in the literature on the physicochemical properties, protein structure, and digestion of different milks. Different protein structures influence the formation of aggregates during the digestion process, which directly contributes to efficient or rapid protein digestion (Boland and Singh, 2019). When breastfeeding is not possible, infant formulas, including those made from goat milk, can be used as alternatives. The European Food Safety Authority (EFSA) reviewed new evidence in 2012 and concluded that goat milk is a suitable protein source for infant and follow-on formula. In the present study, the protein profiles, light microscope images, particle sizes, ACE and antioxidant capacity (%) of human milk, cow milk, and goat milk were examined to evaluate their behaviours during the in vitro digestion protocol. The gastric phase initiates protein digestion, which concludes in the gastrointestinal phase, making protein the most crucial nutrient for infant nutrition. During this process, the type of milk, which is the primary component of infant nutrition, should be assessed at different stages of digestion (post-gastric and post-gastrointestinal phases). In addition to gastric digestion studies reported in the literature (He *et al*., 2022), the identification of protein structures after the gastrointestinal digestion process was considered a necessity. Thus, the behaviours of cow and goat milk, considered as alternatives to human milk, were evaluated before digestion (0. minute), after gastric digestion (60.minute), and after gastrointestinal digestion (120.minute).

Materials and Methods

Materials

Cow and goat milk was purchased as the most readily available UHT cow and goat milk in Izmir local markets. For human milk, a pool was created from the milk collected from six healthy mothers, aged 28–35, within the first 6 months of lactation. The milk was stored in sterile packaging at −20°C immediately after milking and preserved until the day of analysis.

Methods

Milk characterisation

The pH values of human milk, cow milk, and goat milk were measured using a Hanna HI 83141 pH meter (Hanna Instruments, Jud. Cluj, Romania). The total dry matter and ash contents were determined according to AOAC standards. In addition, the fat (Renner, 1993) and protein (AOAC, 2003) contents of the milks were analysed in human milk, cow milk, and goat milk.

Simulated gastrointestinal digestion

Infant digestion occurs under milder physiological conditions than adult digestion, characterized by different pH levels, limited enzyme activity, and different digestive fluid compositions. The simulated infant gastrointestinal digestion was performed using a standardised static in vitro digestion method with minor modifications developed by Menard *et al*. (2018). Enzyme activities were determined according to Minekus *et al*. (2014), and this protocol was modified at some stages.

According to Minekus *et al*. (2014), if the sample does not contain starch in the in vitro digestion process, the digestion simulation can continue without addition in the oral phase. For this reason, it is appropriate to continue the gastric and intestinal phase without adding α-amylase for different milk samples, as they do not contain starch. The main stage for the breakdown and digestion of proteins takes place in the stomach (Rodríguez, León, & Bustos, 2022). For this reason, the sample taken after the gastric stage should be carefully evaluated for protein digestion.

For the infant digestion process, simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared and stored at +4°C. SGF, containing 94 mM sodium chloride, 13 mM potassium chloride, and 268 U/mL pepsin (Sigma), was adjusted to pH 5.3 at 37°C in a water bath before mixing with milk at a ratio of 37:63. The gastric digestion lasted 60 min in the bath with continuous shaking at level 3. SIF comprised 164 mM sodium chloride, 10 mM potassium chloride, 85 mM sodium bicarbonate, 3 mM calcium chloride, 3.1 mM bile salts, and porcine pancreatin (90 U/mL lipase activity), and was adjusted to pH 6.6 at 37°C in a water bath before mixing with milk at a ratio of 37:63. Human milk, cow's milk, and goat's milk samples were digested and sampled before digestion (0 min), after gastric digestion (60 min), and after gastrointestinal digestion (120 min). Milk protein hydrolysis was stopped by the addition of Pefabloc (5 mM final concentration). The samples were centrifuged at 9000 rpm for 10 min, and the supernatant fraction was filtered and stored at −18°C for further analyses.

Samples prepared with equal protein concentrations (1 mg/ mL) and 10 µl of sample were loaded into wells prepared in the Mini-Protean II system (Bio-Rad Laboratories, Richmond, CA, USA). Electrophoresis was performed for approximately 2 h at a constant voltage of 100 mV until the bromophenol blue dye reached the bottom of the gel. After staining and destaining were completed, the gels were scanned using Bio-Rad Image Lab software with the VersaDoc MP 4000 Molecular Digital Imaging System (Bio-Rad).

Antioxidant and angiotensin converting enzyme (ace) inhibitory activity

The antioxidant activity was assessed employing the CUPRAC assay under a pH 7.0 condition, enabling the evaluation of both hydrophilic and lipophilic antioxidant compounds (Apak *et al*., 2004). Total antioxidant capacity was expressed as ascorbic acid equivalents, calculated by considering the dilution factors based on a calibration curve generated within the concentration range of 20–100 µmol/ml of ascorbic acid.

The Angiotensin Converting Enzyme (ACE) inhibitory activity was determined spectrophotometrically at 340 nm using a UV–visible spectrophotometer (ThermoScientific, Multiskan Sky, Finland), according to the procedure outlined by Shalaby *et al*. (2006).

Particle size distribution

The Z-average diameter is a value calculated based on density. The Z-average diameter of casein micelles is defined according to Roy *et al*. (2020). Diluted samples of human, cow, and goat milk were filtered through a 0.45 µm syringe filter to remove large particles before digestion. Measurements were taken for samples collected before digestion and at the 60th and 120th minute stages of digestion. The samples were used to conduct potential measurements using a Zeta sizer instrument (NanoZS, Malvern Instruments Ltd., Malvern, UK) at 20°C. All zeta potential measurements were performed three times, and the average values were used.

Light microscopy

Light microscopy imaging was conducted to provide a high-resolution examination of the microstructures of human milk, cow's milk, and goat's milk during the in vitro digestion stages. Changes in protein structures were analysed using 0.1% toluidine blue before digestion, after gastric digestion, and after gastrointestinal digestion. Microscopic images were captured using an Olympus CX31 microscope fitted with an Olympus DP25 camera (Morell *et al*., 2017).

Statistical analysis

A one-way analysis of variance (one-way ANOVA) was performed to determine the differences between the samples of human milk, cow milk, and goat milk using SPSS version 22.0 statistical analysis software (SPSS Inc., Chicago, Illinois). Significant differences were further evaluated using the Duncan multiple comparison test at the $p<0.05$ level. The analyses were performed in two parallel sets with three replicates, and the results were presented as mean ± standard deviation.

Results and Discussion

When human milk is insufficient or unavailable, there is a need for alternative food sources. While goat milk, with its rich composition, serves as an alternative to human milk, its limited availability (due to the fact that it is not an industrial product) and its high cost often lead individuals to turn to cow milk. Cow milk, in contrast, is relatively cheaper and an industrial product compared to goat milk. Identifying the protein profile of food is especially important during this period of rapid growth and development. Evaluation of the protein profiles of both human milk and its alternatives, cow and goat milk, during the digestive stages of infants will contribute to the interpretation of alternatives in infant nutrition.

Milk components

Table 1 presents the pH values and basic components of human milk, cow milk, and goat milk. The pH values measured before digestion were found to be 6.66 for human milk, 6.79 for cow milk, and 6.71 for goat milk. In another study, the pH values for human milk, cow milk, and goat milk were reported as 7.15, 6.58, and 6.47, respectively (El-Hatmi *et al.*, 2015). The total dry matter content was found to be 11.75%, 13.62%, and 11.00% for human milk, cow milk, and goat milk, respectively. The protein, fat, and ash contents in human milk were found to be relatively lower compared to cow and goat milk, and the difference was statistically significant (P ≤ 0.05). Kumar and Sharma (2016) reported that the ash content of cow and goat milk was 0.7–0.8%, fat content was 3.6–3.8%, and protein content was 3.2–3.4%. Compared to these data, the ash content was similar to our study, while the fat and protein contents were found to be higher.

The protein content of human milk was found to be 0.59%, while in cow and goat milk the values were 2.45% and 2.02%, respectively. In a study by El-Hatmi *et al.* (2015), the protein content of human milk was reported as 1.41%, while the corresponding values for cow and goat milk were 2.59% and 2.49%, respectively, and it was noted that the protein content of human milk was significantly different from that of the other milks (p<0.05).

According to Table 1, it should not be assumed that HM is the poorest in all nutrients. HM has a different macronutrient distribution—typically higher lactose and lower protein—so. while it may be equal to or higher than GM in dry matter (which combines protein, fat, carbohydrates, ash, vitamins), the protein/fat fractions within the dry matter are lower. In cow, goat, and human milks, the dominant non-protein/non-fat fraction of DM is lactose; ash (minerals) is modest, and vitamins constitute a trace mass fraction. Accordingly, a low (protein + fat)/DM ratio primarily indicates a lactose-dominant matrix, with the remainder constitute ash and trace vitamins.

Milk proteins are defined as the most important nutrients for infant nutrition. The composition of human milk differs from that of cow and goat milk. In particular, the protein composition emerges as an important factor in the selection of alternative milks to human milk. In the literature, goat milk is referred to as the closest alternative to human milk, with many health-promoting properties highlighted (Kumar and Sharma, 2016; Dhasmana, 2022).

SDS-PAGE profile

During infancy, proteins obtained from human milk serve as the fundamental building blocks for growth and development, supporting the body's construction and repair mechanisms. The type and composition of the milk consumed are important not only for meeting the nutritional needs of the organism but also in terms of protein functionality. Studies on milk proteins have reported the following molecular weights: αs1-casein 22–23 kDa, αs2-casein 25 kDa, β-casein 24 kDa, κ-casein 19 kDa, β-lactoglobulin 18 kDa, α-lactalbumin 14 kDa, and bovine serum albumin 66 kDa (Eigel *et al.*, 1984). Figure 1 shows protein profile changes during in vitro gastrointestinal digestion (0, 60, and 120 min) of human, cow, and goat milk, respectively, as analysed by SDS-PAGE. The protein composition of milk varied among human, cow, and goat milk digestions (Figure 1, Bands 2, 5, and 8).

In the literature, β-casein, the predominant casein protein in both goat and bovine milk, demonstrate notable stability during the gastric phase of an in vitro digestion model designed to simulate infant digestion (O'Callaghan *et al.*, 2011). In addition, human milk, known as albumin-rich milk, exhibits different behaviour. Whey proteins resist stomach conditions, but undergo intensive hydrolysis during the intestinal phase of digestion, as observed in infant formula studies (Gallier *et al.*, 2015). All three samples show progression of protein digestion during the gastric digestion phase. This is evidenced by the disappearance of bands associated with intact proteins and the decrease in their intensity, which occurs with the reduction in pH. After the gastric digestion (y), which is identified in Bands 3, 6, and 9, confirming pepsin action on undigested proteins.

Table 1. Components of human milk, cow milk, and goat milk.

Sample	pH	Dry matter (%)	Ash (%)	Fat (%)	Protein (%)
HM	6,66 ± 0,01^{C}	11,75 ± 0,20^{B}	0,22 ± 0,06^{C}	2,20 ± 0,00^{B}	0,59 ± 0,02^{B}
CM	6,79 ± 0,00^{A}	13,62 ± 0,03^{A}	0,69 ± 0,04^{B}	3,30 ± 0,00^{A}	2,45 ± 0,01^{A}
GM	6,71 ± 0,00^{B}	11,00 ± 0,02^{B}	0,78 ± 0,01^{A}	3,00 ± 0,00^{A}	2,02 ± 0,04^{A}

A, B, C Different letters indicate significantly different values in the same column (P ≤0.05).
HM: Human milk; CM: Cow milk; GM: Goat milk.

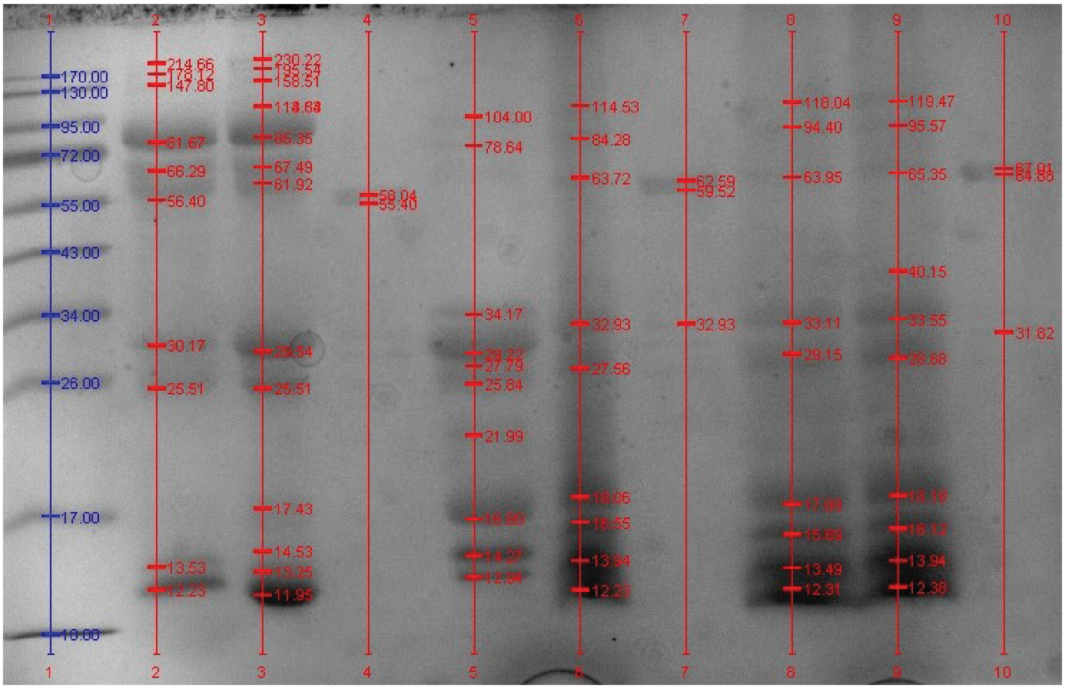

Figure 1. ***In vitro*** **infant digestion profile of human milk, cow milk, and goat milk.**

Bands: 1:Marker; 2:Undigested human milk; 3:60. min. gastric digestion of human milk; 4:120. min. gastrointestinal digestion of human milk; 5:Undigested cow milk; 6:60. min. gastric digestion of cow milk; 7:120. min. gastrointestinal digestion of cow milk; 8:Undigested goat milk; 9:60. min. gastric digestion of goat milk; 10:120. min. gastrointestinal digestion of goat milk.

Notably, α-lactalbumin, a significant protein in human milk, constitutes approximately 17–28% of the total protein content, whereas its proportion in cow milk is markedly lower, accounting for only 3.0–3.5% of the total protein (Lin *et al*., 2024). In a study, it was stated that β-Lg and α-La in cow milk are resistant to human gastric and intestinal enzymes (Inglingstad *et al*., 2010). However, in the present study, the β-Lg and α-La protein structures observed in cow and goat milk after gastric digestion (bands 6, 9) were not detected after gastrointestinal digestion (bands 7, 10). This result suggests that the β-Lg and α-La present in cow and goat milk may undergo hydrolysis in the infant digestion model. β-casein, the predominant casein protein in both human and bovine milk, has been reported to show remarkable stability during the gastric phase of an in vitro digestion model designed to simulate infant digestion. In human milk, significant protein structures such as lactoferrin, serum albumin, β-casein, and α-lactalbumin were observed after gastric digestion (band 3) but were not detected after the gastrointestinal digestion phase (band 4). This finding indicates that the digestion process of human milk proteins is largely completed after the intestinal phase. High molecular weight proteins observed during the gastric phase of digestion in human milk (band 3) were not detected in cow and goat milk after gastric digestion (bands 6, 9).

Angiotensin converting enzyme (ACE) inhibitory and antioxidant activity

Table 2 presents the ACE inhibition activities of human milk, cow milk, and goat milk at different stages of simulated digestion. Initially, cow milk and goat milk exhibited significantly higher ACE inhibitory activities compared to human milk ($P \leq 0.05$). Throughout digestion, an overall increase in ACE inhibition was observed in all samples, reflecting the enzymatic release of bioactive peptides during gastrointestinal processes.

The highest post-digestion ACE inhibition (82.86%) was recorded in cow milk after 120 minutes, indicating its strong potential for antihypertensive effects. Goat milk also demonstrated a notable increase, although its final ACE inhibition was lower than cow milk but higher than human milk. In contrast, human milk showed a modest increase in ACE inhibition, reaching only 50.04% after full digestion, significantly lower than both cow milk and goat milk ($P \leq 0.05$). Significant differences were also seen at different stages of digestion, particularly emphasising the effect of gastric and intestinal digestion. These results suggest that cow milk proteins may release greater amounts or more potent ACE inhibitory peptides during digestion compared to goat and human milk. This finding highlights the potential of products derived from cow's

Table 2. ACE Inhibition Activity (%).

Sample	Before digestion (0. minute)	Gastric Digestion (60. minute)	Gastrointestinal Digestion (120. minute)
HM	45.04 ± $0.02^{B,Y}$	49.52 ± $0.02^{B,X}$	50.04 ± $0.03^{C,X}$
CM	68.26 ± $0.01^{A,Z}$	71.20 ± $0.00^{A,Y}$	82.86 ± $0.03^{A,X}$
GM	68.00 ± $0.01^{A,Z}$	70.62 ± $0.02^{A,Y}$	74.20 ± $0.01^{B,X}$

A, B, C Different letters indicate significantly different values in the same column (P≤0.05).
X, Y, Z Different letters indicate significantly different values on the same line (P≤0.05).
HM: Human milk; CM: Cow milk; GM: Goat milk.

Table 3. Antioxidant activity values (mMeq Ascorbic acid/µg).

Sample	Before digestion (0. minute)	Gastric Digestion (60. minute)	Gastrointestinal Digestion (120. minute)
HM	0.18 ± $0.03^{C,Z}$	0.29 ± $0.02^{C,Y}$	0.77 ± $0.05^{C,X}$
CM	0.76 ± $0.01^{A,Z}$	0.88 ± $0.01^{A,Y}$	1.06 ± $0.08^{A,X}$
GM	0.52 ± $0.05^{B,Z}$	0.74 ± $0.06^{B,Y}$	0.96 ± $0.03^{B,X}$

A, B, C Different letters indicate significantly different values in the same column (P≤0.05).
X, Y, Z Different letters indicate significantly different values on the same line (P≤0.05).
HM: Human milk; CM: Cow milk; GM: Goat milk.

milk in the development of functional foods aimed at regulating blood pressure.

The antioxidant activity observed in milk and dairy products is attributed to various components within their composition, such as proteins, bioactive peptides, and enzymes, each exhibiting distinct antioxidant capacities. Table 3 summarises the antioxidant activities of human milk, cow milk, and goat milk during simulated digestion. Before digestion, cow milk exhibited the highest antioxidant activity (0.76 mMeq ascorbic acid/µg), followed by goat milk and human milk, with statistically significant differences observed between all samples (P ≤ 0.05). As digestion progressed, antioxidant activities increased in all samples, likely due to the release of antioxidant peptides and bioactive compounds through proteolytic action. Human milk demonstrated superior enhancement in antioxidant activity during digestion, highlighting its potential as a functional food ingredient for health promotion.

After 60 minutes of gastric digestion, cow milk maintained its superior antioxidant activity, whereas goat milk showed a moderate increase, and human milk remained comparatively lower. By the end of gastrointestinal digestion (120 minutes), cow milk again demonstrated the highest antioxidant capacity (1.06 mMeq ascorbic acid/µg), while goat milk and human milk followed. The antioxidant activity of all milk types increased significantly throughout the in vitro digestion process (P ≤ 0.05). Although human milk (HM) initially exhibited the lowest antioxidant capacity, it showed the highest relative increase after 120 minutes of digestion, rising more than fourfold compared with its initial value. This pronounced increase suggests that enzymatic hydrolysis during gastrointestinal digestion may release a higher proportion of antioxidant peptides or bioactive components from human milk proteins.

When ACE inhibition and antioxidant activity results are considered together, the increases observed in all samples throughout digestion suggest that proteolytic processes enhance the release of bioactive peptides with health-promoting properties. Overall, the findings suggest that both cow and goat milk can serve as valuable raw materials for the development of functional foods aimed at improving cardiovascular and antioxidant health. Cow milk and goat milk can be identified as important sources when human milk is inaccessible or limited. Further research is recommended to identify and characterise the specific peptide sequences that contribute to these bioactivities.

Light microscopy imaging

The light microscopy images taken during the digestion stages are shown in Figure 2. Imaging conducted at different stages of digestion is important for understanding the potential preference of human milk, cow milk, and goat milk in infant nutrition. In human milk, at 0 minutes, the protein particles are observed to be homogeneously distributed and of small size. After 60 minutes, protein breakdown has begun in human milk, and the particles

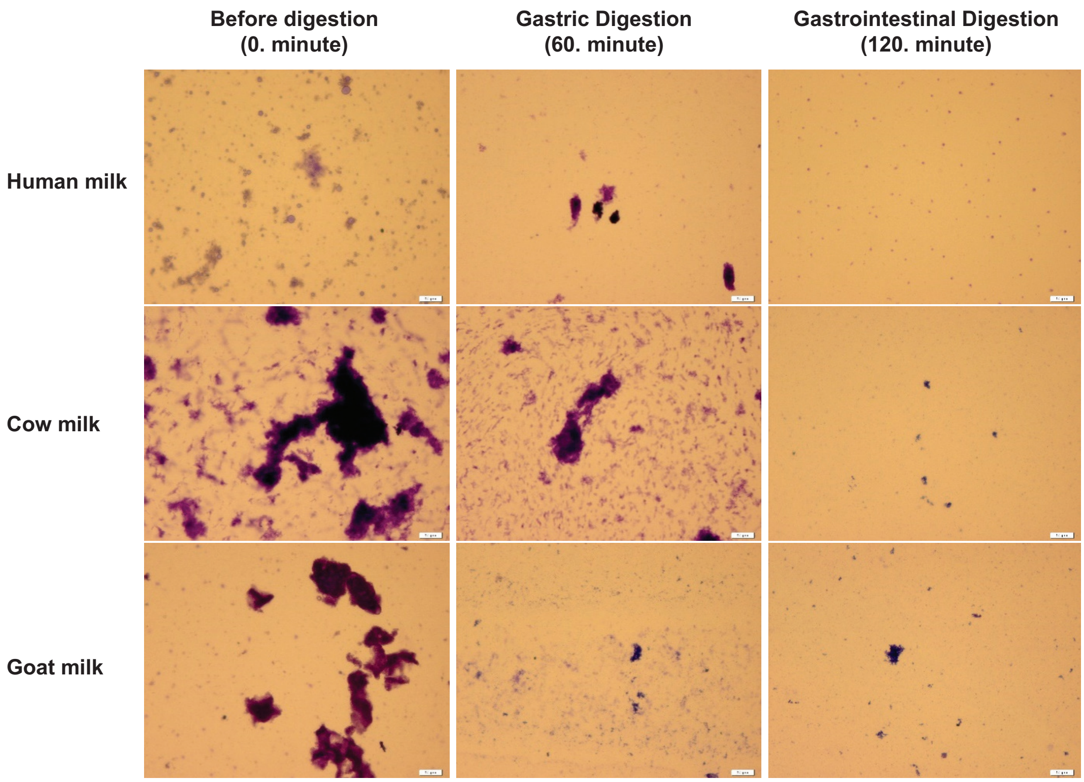

Figure 2. Light microscopy images of protein profiles in human milk, cow milk, and goat milk (Scale bars=50 μm.) HM: Human milk; CM: Cow milk; GM: Goat milk.

have shrunk and become less dense. By 120 minutes, the protein particles have completely dispersed or reached smaller sizes suitable for digestion. This indicates that human milk easily adapts to the gastrointestinal digestion simulation. In cow milk, larger and denser protein clusters are observed before digestion, and by 60 minutes, breakdown begins; however, the distribution occurs more slowly compared with human milk.

Even though breakdown continues in the cow milk samples taken after 120 minutes, the post-digestion particles have not completely broken down. This finding suggests that cow milk is more difficult to digest after gastrointestinal digestion. In goat milk, the protein particles before digestion are in a more homogeneous state compared with cow milk. After gastric digestion, goat milk samples show a model closer to that of human milk, and by the time gastrointestinal digestion is complete, most of the particles have been digested, with a homogeneous distribution of the remaining particles. This finding supports the idea that goat milk completes the digestion process more efficiently than cow milk and exhibits a digestion profile similar to that of human milk. The data obtained suggest that human milk is the easiest to digest and the most suitable for infant nutrition during the in vitro infant digestion simulation process. Furthermore, in cases of human milk deficiency or unavailability, cow milk, which is commonly preferred for infant formula production, is more difficult to digest compared with human milk and goat milk due to the presence of larger protein particles.

Nguyen *et al.* (2015) demonstrated that dairy proteins in infant formulas form large aggregates at the onset of simulated digestion; however, after 1 hour of proteolysis in the stomach, these aggregates are reduced in size compared with the initially formed structures.

Human milk exhibits microstructural differences compared with cow and goat milk. Protein aggregates appear smaller during the digestion stages, likely due to the lower casein content in human milk. Following gastric digestion, smaller aggregates were observed in human milk and goat milk compared with cow milk (Figure 2). Additionally, after completion of gastrointestinal digestion, large aggregates were still present in cow milk, unlike the homogeneously distributed aggregates in human milk. Goat milk, on the other hand, completed gastrointestinal digestion with smaller aggregate clusters compared with cow milk but still differed from human milk. This phenomenon is thought to be due to the formation of looser curds in goat milk during digestion, likely influenced by pH changes (Sun *et al.*, 2019). The obtained data suggest that, although cow milk is more readily accessible, its digestion characteristics deviate further from human milk compared with goat milk.

In a study in which proteins were microscopically imaged during digestion, the behaviour in infant and adult digestion models was evaluated. It was reported that whey proteins generally undergo hydrolysis in the intestinal phase and show greater resistance to gastric digestion, especially in human milk, compared with caseins (Zhao *et al.*, 2023).

Particle size

Determining the particle size distribution provides insight into the formation of aggregates and their breakdown during simulated gastric digestion. When the particle size values were examined, the highest value was observed in human milk before digestion, while protein digestion starting in the stomach is thought to have an effect on particle size in cow and goat milk. Throughout ongoing digestion, an increase was observed in cow and goat milk, whereas a decrease was noted in human milk. With its relatively lower protein content compared with the other milks, human milk showed a decrease in particle size during the gastric phase (1219.30 nm), followed by a further decrease (202.63 nm).

In a study by He *et al.* (2020), the particle sizes of samples consisting of human milk and infant formulas were examined at different pH values. The researchers reported that the digestion behaviour of human milk differed from that of both goat milk- and cow milk-based infant formulas. Furthermore, the researchers noted that pH value influences the formation of protein aggregates.

Cow milk contains a higher protein content compared with human milk. Its composition is approximately 80/20 casein/whey, and an increase in particle size values is observed due to protein digestion that begins in the gastric phase. Similar to cow milk, goat milk also showed an increase in particle size during the digestion process. The decrease in pH during the gastric digestion phase leads to the formation of protein aggregates, and the size of these aggregates varies depending on the type of milk ($p \leq 0.05$).

A study suggested that goat milk contributes to more efficient or rapid protein digestion compared with cow milk due to the formation of softer and looser aggregates during gastric digestion (Boland and Singh, 2019).

However, the results obtained in the present study indicate that the behaviour of these aggregates during the infant digestion simulation was statistically similar. It was observed that the aggregates represented in human milk after gastric digestion were smaller compared with those in cow and goat milk, and this difference was statistically significant ($p \leq 0.05$). In the continuing stages of digestion, an increase in clustering within the protein structures was observed (Table 4). The significant increase observed in cow and goat milk exhibited behaviour that was opposite to that of human milk. Goat milk aggregates exhibited a looser and larger structure under infant gastric conditions, consistent with the present findings and previous studies.

Conclusion

This study demonstrates the behaviour of cow milk and goat milk during infant digestive stages when breastfeeding is not possible or is limited; these milks are the fastest and most accessible alternatives. Although human milk is the ideal nutritional source for infants, this study represents the first systematic analysis of the digestive processes of alternative milk sources, especially cow milk and goat milk, excluding infant formula. The results confirm that human milk is the most suitable and easily digestible milk in terms of digestive processes and also show that the particle size of proteins in human milk decreases during gastrointestinal digestion. Considering that these variations may affect the gastrointestinal digestion rate of human milk, further studies are needed to monitor the digestive process minute by minute. Light

Table 4. Particle size of human milk, cow milk, and goat milk (ave. diameter (nm)).

Sample	Before digestion (0. minute)	Gastric Digestion (60. minute)	Gastrointestinal Digestion (120. minute)
HM	1785,30 ± 70,18^{A,X}	1219,30 ± 4,33^{A,Y}	202,63 ± 28,91^{B,Z}
CM	193,23 ± 3,98^{B,Z}	463,90 ± 12,82^{B,Y}	1567,60 ± 82,67^{A,X}
GM	334,13 ± 20,62^{B,Z}	489,27 ± 11,42^{B,Y}	2048,73 ± 37,39^{A,X}

A, B, C Different letters indicate significantly different values in the same column (P≤0.05).
X, Y, Z Different letters indicate significantly different values on the same line (P≤0.05).
HM: Human milk; CM: Cow milk; GM: Goat milk.

microscopy images and changes in particle size for both goat milk and cow milk show distinct profiles compared with human milk. The protein composition and aggregate structures in these milks were evaluated as the main factors determining the speed and efficiency of digestive processes. In particular, cow milk and goat milk entered digestion with larger protein aggregates, while human milk and goat milk were found to be digested more quickly and efficiently after gastric digestion. In conclusion, these results provide a scientific basis for the valorisation of cow and goat milk in the design of bioactively enriched dairy products. Future studies should focus on the isolation, identification, and functional validation of specific peptide sequences responsible for ACE inhibition and antioxidant activities to better understand their mechanisms of action and optimise their applications in the nutraceutical and clinical fields. Additionally, it is the first study to directly evaluate cow and goat milk in combination with human milk in an infant digestion simulation. However, more research is needed to monitor the behaviour of milks other than infant formula and follow-on milk in combination with human milk in infant digestion simulations.

Conflicts of Interests

The author has no relevant financial interests to disclose.

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