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Proteome Analysis of Human and Goat Colostrum: A Closer Look at Whey Fractions

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ABSTRACT

Background/Purpose: Human colostrum, the first form of milk produced by mammary glands, is crucial for newborn development. Nowadays, there is a great interest in finding alternative colostrum from different species to replace the extremely scarce human colostrum. In this study, we investigated the proteomic profiles of whey fractions of human and goat colostrum samples to understand the proteomic differences and gain insight into the potential functions of these proteins.

Methods: Proteomic profiles of human (n=6) and goat (n=6) colostrum that were collected at the early stages of lactation were investigated using two-dimensional difference gel electrophoresis (DIGE). Spot intensity differences were detected and spots were identified by MALDI-TOF/TOF mass spectrometry. Functional annotation analyses were performed.

Results: In total, 533 spots were detected and identified in human and goat colostrum samples. Immunoglobulin, casein, lactoferrin, lactoglobulin, albumin, lactotransferrin, and lactalbumin proteins were found to be abundant. Low abundance proteins such as α1-antitrypsin, cathelicidin, galectin-3-binding protein, lactadherin, tenascin, and apolipoprotein J were also detected. Functional annotation analysis showed that human colostrum proteins were commonly involved in the phagosome, complement and coagulation pathways, and disease-related pathways.

Conclusion: Our results provide a preliminary proteomic comparison between human and goat colostrum samples. The proteins detected in the whey fractions of human and goat colostrum showed a remarkable number of common proteins. Moreover, human colostrum showed disease-related pathway enrichments and further suggests the role of passive immunization that might protect the newborn from diseases.

Keywords: 2-DIGE, goat colostrum, human colostrum, MALDI-TOF/TOF, whey proteins

İnsan ve Keçi Kolostrumunun Proteomik Analizi: Whey Fraksiyonuna Yakından Bakış

Giriş/Amaç: Meme bezleri tarafından üretilen sütün ilk formu olan insan kolostrumu, yenidoğan gelişimi için çok önemlidir. Günümüzde, miktar olarak az olan insan kolostrumunun yerine farklı türlerden alternatif kolostrum bulmaya yönelik büyük bir ilgi mevcuttur. Bu çalışmada, kolostrumdaki proteomik farklılıkların ve bu farklılığa sebep olan proteinlerin potansiyel işlevlerinin anlaşılması için, insan ve keçi kolostrum örneklerinin whey fraksiyonlarının proteomik profilleri araştırılmıştır.

Yöntemler: Laktasyonun erken döneminde toplanan insan (n=6) ve keçi (n=6) kolostrum örneklerinin proteomik profilleri iki boyutlu diferansiyel jel elektroforezi (DIGE) kullanılarak incelenmiştir. Protein spot yoğunluğu farklılıkları tespit edilmiş ve bu spotlar MALDI-TOF/TOF kütle spektrometresi ile tanımlanmıştır. Proteinlerin fonksiyonel anotasyonları analiz edilmiştir.

Bulgular: İnsan ve keçi kolostrum örneklerinde toplam 533 protein spotu tespit edilmiş ve tanımlanmıştır. İmmünoglobulin, kazein, laktoferrin, laktoglobulin, albümin, laktotransferrin ve laktalbumin proteinlerinin bol miktarda bulunduğu görülmüştür. Bunun dışında, α1 antitripsin, katelisidin, galektin-3 bağlayıcı protein, laktadherin, tenascin ve apolipoprotein J gibi düşük yoğunluklu proteinler de tespit edilmiştir. Fonksiyonel anotasyon analizine göre, insan kolostrum proteinlerinin yaygın olarak fagozom, kompleman ve pıhtılaşma yolları ve hastalıkla ilgili yolaklarda yer aldığı gösterilmiştir.

Sonuçlar: Elde edilen bulgular ile insan ve keçi kolostrum örnekleri arasında bir ön proteomik karşılaştırma yapılmıştır. İnsan ve keçi kolostrumunun whey fraksiyonlarında yapılan bu proteomik çalışma ile çok sayıda ortak proteinin varlığı gösterilmiştir. Ancak, insan kolostrumunda çeşitli hastalıklarla ilişkili yolakların ön plana çıktığı ve bu durumun yenidoğanı hastalıklardan koruyabilecek pasif bağışıklıkla ilgili olabileceği düşünülmüştür.

Anahtar Kelimeler: 2-DIGE, keçi kolostrumu, insan kolostrumu, MALDI-TOF/TOF, whey proteini

Human milk is the most suitable nutrition for a newborn that provides growth, developmental factors, bioactive components, and immunity for a newborn's survival in short and long lifetime periods (1, 2) . The high level of immunoglobulin A (IgA) in human colostrum protects newborns from potential infections (3) . Additionally, colostrum supports the formation of a normal and healthy gut microbiome in the infant.

Human mature milk and colostrum mainly consist of high abundant proteins (HAPs), such as alpha-lactalbumin and lactoferrin (4), whereas complement factors, acute phase proteins, anti-microbial proteins, peptides, and cytokines are known as low abundant proteins (LAPs) and commonly found in whey fraction (5). Low abundant proteins are physiologically important since they protect against pathogens and other environmental challenges (6) and participate in the passive immune transfer, and are fundamental for the growth and development of newborns (4).

Recently, there is an ongoing research interest in using alternative sources of colostrum to replace extremely scarce human colostrum. The composition of colostrum is known to be different in animals and humans. Yet, cow milk-containing products are predominantly used in newborn formulas and protein supplements. However, cow milk allergy is frequently seen in infants (7). Due to the compositional changes and allergenic properties, goat milk has been recommended as a substitute for patients allergic to cow milk (8). Still, studies assessing the potential of goat colostrum to replace human colostrum remains limited.

It is well-known that the milk fat globule membrane (MFGM) fraction of the colostrum does not vary (9); whereas LAPs in the whey fraction seem to alter during lactation (10). Therefore, it is important to assess LAPs in colostrum to gain deeper information about their functionality. A substantial number of papers were published about the nutritional importance and complex molecular components of milk over the years (11). Recently, proteomic methodologies in milk/colostrum research are becoming increasingly important to discover the functional contribution of human milk proteins or milk proteins from different species to infants' development (12). In this concept, the whey fractions and MFGM compositions of goat colostrum and mature milk have been extensively studied (13,14). However, the comparison of the whey fraction proteomic profiles of human and goat colostrum remains inconclusive.

In the current study, we investigated the proteomic profiles of the whey fractions of human and goat colostrum using two-dimensional difference gel electrophoresis (DIGE) followed by mass spectrometric analysis. Proteomic analysis of human and goat colostrum samples is important to detect proteomic pathways and determine over-and/or under-represented LAPs in each group.

Material and Methods

Sample Collection

Human colostrum samples were collected from subjects who agreed to sign an informed consent form from Zeynep Kamil Research and Training Hospital Istanbul-Turkey, a state hospital that specialized in obstetrics and gynecology. Mothers who delivered singleton-term newborns at 38-41 weeks of their pregnancy participated and mothers with illnesses; such as cold, mastitis, and flu were excluded from the study. Six colostrum samples were collected up to 24h of lactation by manual expression or manual breast pump into 50 ml polypropylene containers. Goat colostrum (GC) samples were obtained from Saanen breeds from a family farm in Izmir-Turkey. Six samples were obtained from healthy goats on their first day, 2h after their partum, by manual expression into 50 ml polypropylene containers. All colostrum samples were stored at –80°C until analysis. The study was approved by the Acibadem University Ethical Committee (ATADEK-2013-507).

Protein Extraction

Colostrum samples were centrifuged at 15.000xg for 10 min at +4°C and the cream layer at the top was removed. Skimmed milk was collected as a supernatant. Protease inhibitor mix of 10 µl (100X, (GE Healthcare) was added to prevent any protein degradation. Samples were then ultracentrifuged using a swinging bucket rotor (SW40ti) at 110.000xg for 1 h at +4°C (Beckman Coulter, optima L-90K ultracentrifuge). Whey fraction was collected as a supernatant and the protein concentration was determined by a 2-D Quant kit (GE Healthcare).

Proteomics Analysis

Human colostrum and GC samples were labeled with three types of fluorescent cyanine dyes (Cy2, Cy3, and Cy5) for the DIGE method. Human and goat samples were divided into two groups. The first group included H1, H2, H3 as human and G4, G5, G6 as goat samples and were labeled with Cy3. The second set included H4, H5, H6 as human and G1, G2, and G3 as goat samples which were then labeled with Cy5. Internal Standard (IS), generated

by pooling aliquots of 12 samples including entire goat (n=6) and human samples (n=6), was labeled with Cy2. All six gels contained one Cy3-labeled sample, one Cy5-labeled sample, and one Cy2-labeled internal standard.

Isoelectric focusing was performed by an Ettan IPGphor 3 system (GE Healthcare) using strips (pH 3-10, 24 cm, NL) that were rehydrated overnight with the labeled samples diluted in a solution consisting of 7M Urea, 2M Thiourea, 0.5% CHAPS, 0.6% Destreak Reagent, and 2% Servalytes as rehydration buffer. Separation in the first dimension was 21h with a total of 90 kVh. The strips were equilibrated for 15 minutes with equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris (pH 8.8), and 1% dithiothreitol (DTT)). Then the same equilibration buffer including 2.5% iodoacetamide rather than DTT was applied for another 15 minutes. The horizontal electrophoresis system (Serva-HPE) was used for the separation in the second dimension with non-fluorescent 12.5% gels. The temperature was set at 15 °C and the running conditions were as: 100V, 30min; 200V, 30 min; 300V, 10min. After 70 minutes run, strips were removed from the gel, and the process was completed by working at 220 V, overnight, and 1000V, 3h. Gels were then fixed in 15% ethanol for 2 hours.

Typhoon FLA 9500 Gel Imaging Scanner (GE Healthcare) was used to visualize protein spots. Spot detection and quantitative analysis were performed for all the six gels based on their fluorescence signals using DeCyder 2D (V7.0, GE Healthcare). Selected protein spots (ratio >1.5 and $p<0.01$) were picked with a robotic system (Ettan Spot Picker, GE Healthcare) and transferred to 96-well plates. Spots were then treated with an EVO 2 liquid handling workstation (TECAN). Spots were washed twice for 20 minutes with 50 mM ammonium bicarbonate in 50% methanol. Spots were then dried with 75% acetonitrile (2 times, 20 minutes) before being incubated with 40 ng trypsin in 20 mM ammonium bicarbonate at 37°C for 6 hours. The peptides were eluted from the gel and subjected to further analysis using a mass spectrometry method.

MALDI TOF/TOF Analysis

Dry peptides were solubilized in 2 µl 50% acetonitrile with 0.1% TFA. Following solubilization, 0.7 µl of peptide sample were spotted on a MALDI target with an equal volume of 7mg/ml α–cyano–4– hydroxycinnamic acid in 50% acetonitrile with 0.1% TFA. Mass spectra were acquired on a 5800 MALDI TOF/TOF analyzer (ABsciex) in a positive reflector mode. The MALDI-TOF/TOF spectra were searched against a database composed of the entries of the "homo sapiens" (txid9606) and "capra" (txid9922) taxonomies from the NCBI database, downloaded on the 24th of January 2017 and containing 1118538 sequences, using Protein Pilot (ABsciex) with MASCOT v2.6 (Matrix Science, Boston, MA) as the search engine. The search parameters were; enzyme: trypsin, the maximum number of miscleaveage allowed: 2, peptide mass tolerance: ±100 ppm, and fragment mass tolerance: w±0.5Da for MS/MS fragments. Additionally, Carbamidomethyl (C) was set as fixed modification and Dioxidation (W), Oxidation (HW), Oxidation (M), and Trp -> Kynurenin (W) were variable modifications. All identifications were manually validated. Unless noted, only proteins with a minimum of 2 peptides identified with MASCOT identity scores greater than 30 were reported.

Statistical Analysis

For each detected spot, the student's t-test and average signal ratios, automatically calculated by Decyder software, were used for spot comparison. For spots having single protein identifications, the initial filtering was based on the ratio (ratio>1.5) and p-value ($p<0.01$) obtained from DeCyder software. Next, the separation into two groups was performed as follows: (i) if ratio > 0, the group was assigned as GC, (ii) if ratio < 0, the group was assigned as HC. Keratin proteins and proteins with no matching gene symbols were excluded from the statistical approaches. The distinct groups of proteins were then used for functional annotation analyses using the DAVID tool (https://david.ncifcrf.gov/). Multiple protein identifications within the same spots were further evaluated by Kyoto Encylopedia of Genes and Genomes (KEGG) pathway analysis to assess the affected pathways.

Results

Common Proteins in Whey Fraction Determined by Two-Dimensional DIGE Analysis

To assess the whey fraction proteomic profile of human and goat colostrum samples, we performed DIGE analysis. In each experiment, every DIGE gel included a Cy2 internal standard of pooled goat and human colostrum proteins. Besides, the proteins labeled with Cy3 and Cy5 were compared in each gel that was normalized with Cy2 labeled samples. A representative Cy5 labeled human colostrum and Cy3 labeled goat colostrum DIGE gels are given in Figures 1 and 2. The master gel was selected based on clarity and the highest number of spots out of six gels. In total, 533 spots were detected and identified in human and goat colostrum samples ($p<0.05$) (Supplementary Figure 1, Supplementary Table 1).

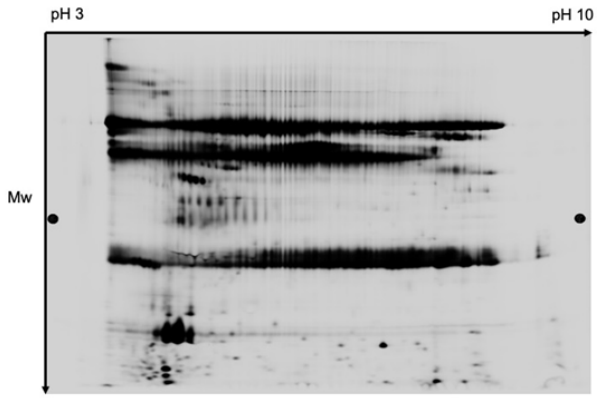

Figure 1. *Representative Cy5 labeled human colostrum DIGE gel*

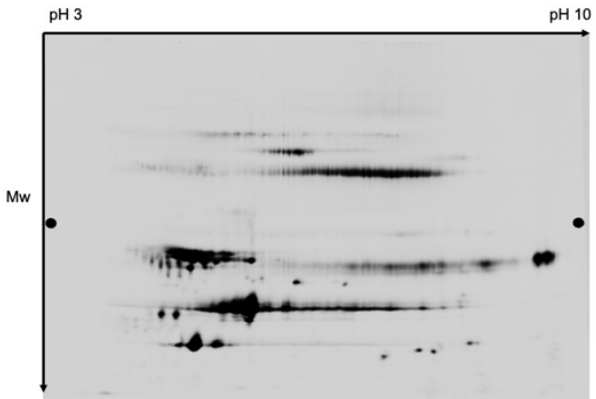

Figure 2. *Representative Cy3 labeled goat colostrum DIGE gel*

Functional Annotation Analyses for Human and Goat Colostrum Samples

For all the proteins identified in human and goat colostrum samples, we determined the gene symbols that were found to be common between HC and GC (Figure 3). For example, *ALB*, *B2M*, *C3*, *IGHG1*, *JCHAIN*, *LALBA*, *LTF*, and *SERPINA3* were the major genes that contributed to the common proteins. Proteins identified in the human colostrum resulted in the enrichment of five KEGG pathways; phagosome, complement and coagulation, pathogenic *E. coli* infection, Legionellosis, *Staphylococcus aureus* infection. Due to the limited number of different proteins identified in goat colostrum, KEGG pathway analysis could not be performed. However, proteins related to the casein fractions and immunoglobulins were the majority of proteins determined in GC.

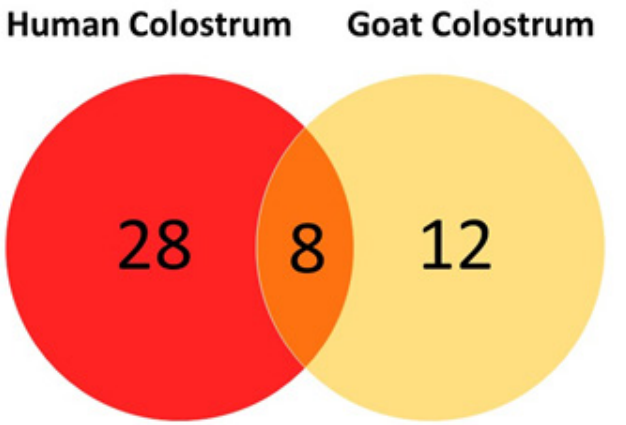

Figure 3. *Venn Diagram of gene symbols that correspond to the proteins identified in human colostrum and goat colostrum.*

Among all the identified proteins in HC and GC, 156 of them were identified in single spots (ratio>1.5 and p<0.01). Sixty-two out of 156 proteins were found to be relatively increased in HC than GC, and 94 out of 156 proteins relatively increased in GC than HC (Supplementary Table 2). Functional annotation analyses were further performed to assess the functional significance of the detected proteins in whey fractions of colostrum samples. Those proteins identified in HC and GC samples were classified based on their "UP-Keywords" (Figure 4). The first three functional categories for proteins in human colostrum were; secreted (20.1%), signal (20.1%), and glycoprotein (20.1%); whereas in goat colostrum samples the first three functional categories were detected as; milk protein (25%), secreted (33.3%), and phosphoprotein (25%). Compared with goat colostrum, human colostrum showed more functional categories. In HC, gene ontology-based biological process pathways were related to homeostatic functions, immune system, and cellular metabolic functions (Figure 5).

Discussion

The benefits of colostrum to the newborn are very well-known, but human colostrum is scarce; therefore, there is a great interest in finding alternative colostrum sources to replace human colostrum. In the present study, we aimed to assess common and differential proteins that were detected in both colostrum types to determine the goat colostrum proteomic coverage of human colostrum. In total, we detected and identified 533 proteins in human and goat whey colostrum samples including;

immunoglobulin, caseins, albumin, lactoferrin, lactotransferrin, and lactoglobulin as HAPs; and α1-antitrypsin, integrin, cathelicidin, galactin-3-binding protein, lactadherin, tenascin, and apolipoprotein J as LAPs.

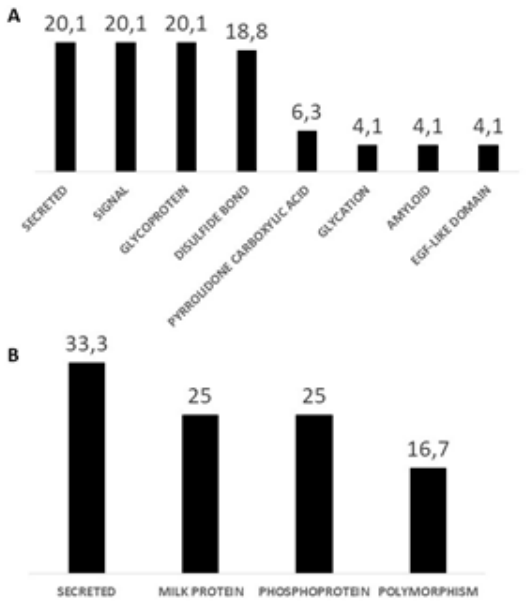

Figure 4. *Functional categories of single spot single protein identifications in the whey fractions of A. Human Colostrum, and B. Goat colostrum using David Tool (UP-Keywords) (ratio>1.5 and p<0.01)*

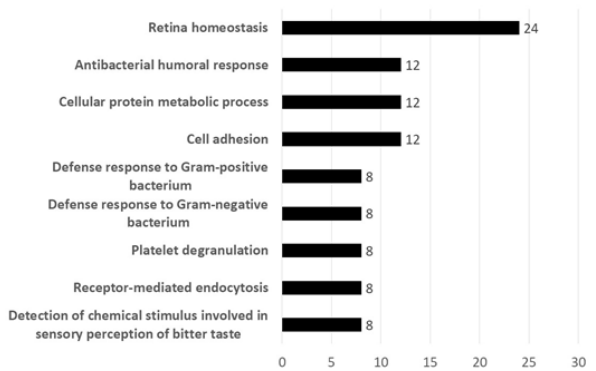

Figure 5. *Gene Ontology classification of human colostrum proteins from the whey fraction based on biological process*

The total protein amount and protein composition change in human mature milk and human colostrum. Additionally, human colostrum had high levels of proteins and a higher proportion of whey fraction when compared with human mature milk. Still, they can share common proteins as well. For example, major and minor proteins like α-1 antitrypsin, α-lactalbumin, carbonic anhydrase 6, cordin like protein 2, galectin-3- binding protein, lactadherin, lactoferrin, prolactin-stimulator protein, and tenascin were common in our human colostrum study and a study in human mature milk (15). Importantly, we detected the aforementioned proteins in goat colostrum; whilst, proteins like α-1 antitrypsin, carbonic anhydrase 6, cordin like protein 2, prolactin-stimulator protein, tenascin, and integrin were not reported in bovine colostrum and milk studies (16).

To find a potential replacement for the scarce human colostrum, it is a priority to deeply understand the protein composition and protein functioning in human colostrum. As an early comprehensive work, Palmer et al. identified 151 proteins in the whey fractions of human colostrum, and 83 out of 151 were reported in human colostrum for the first time (17). Since then, different fractions of human colostrum were investigated using proteomic and phosphoproteomic approaches (18, 19). Similar to our results, proteins involved in complement and coagulation cascades, immune system processes, and signaling pathways were commonly detected in published studies (19, 20). In a human skim colostrum study, metabolism-related proteins such as fructose-bisphosphatase aldolase A were detected and changes in the levels on different days were reported (21). We were not able to detect any specific metabolism-related proteins in human and goat colostrum samples, although proteins in HC were categorized in "cellular and metabolic functions". Detection of no specific metabolism-related proteins might be due to the limitations of the proteomic approach that we used or metabolism-related proteins may not be distinguishable in the time that we collected the colostrum samples. The HAPs, such as IgA, lactoferrin, and human serum albumin, that were detected in human colostrum were in line with the published literature (21). Additionally, we confirmed the presence of these HAPs in goat colostrum as well.

The nutritional benefits of goat milk paved the way for detailed functional assessments of goat colostrum. The protein content is important to further assess the properties of goat colostrum. Yet, there are limited proteomic studies in this field (14).

Recently, Sun et al. showed that proteins involved in extracellular regulated protein kinases (ERK1 and ERK2) signaling and calcium-binding were detected in goat colostrum samples (13). Interestingly, the authors demonstrated that goat colostrum and mature milk proteins were involved in disease-related pathways, like Staphylococcus aureus infection (13). In our study, the detected proteins in human colostrum samples were also enriched to Staphylococcus aureus infection based on the KEGG pathway analysis. However, due to the limited number of proteins identified in goat colostrum, we could not show any enrichment of proteins in the whey fraction of goat colostrum. In the cited study, goat MFGM proteins in the colostrum and mature milk were compared, and both MFGM proteins originated either from colostrum or mature milk showed an enrichment to disease pathways including Staphylococcus aureus infection, pertussis, and legionellosis (13). The enrichment of the disease-related pathways in human and goat colostrum may be the reason for passive immunization that protects the newborn from potential diseases. This is rather critical for ruminants as colostrum is of great importance for the immunization (22). Enrichment of disease-related pathways in human colostrum demonstrated in our study was also in line with the published literature (23). For example, the detection of N-linked glycans and multiple fucosylation products in human MFGM highlighted the potential of preventing infants from infection and disease (23).

In our study, we detected considerable amounts of common proteins in whey fractions of human and goat colostrum, suggesting a relatively high proteomic coverage of human colostrum by goat colostrum. However, an important issue regarding ruminant milk and/or colostrum in humans is the potential allergic reactions they might cause. Food allergies in infants are frequently due to the consumption of cow milk (24). The milk contents of goat and cows are comprised of HAPs including β-lactoglobulin, α-lactalbumin, κ-casein, αS1-casein, and LAPs such as lactoferrin, transferrin, and prolactin (25). However, one case study suggested a role for goat allergy in infants caused by the casein fraction of the goat milk (24). For example, alphaS1-casein levels in ruminant milk can be responsible for allergic reactions in humans (26). Although the whey fraction of goat colostrum and/or milk may not be influential in allergic responses, comprehensive analyses on allergy are required to investigate the potential of goat colostrum to replace human colostrum for newborns.

The content of skim milk (Caseins and whey proteins are named after "skim milk") continuously changes because of the variable whey and casein fraction ratios (9). Whey protein: casein ratio of milk is 80:20 at the beginning, 60:40 at the middle, 50:50 at the end of the lactation period (27). Depending on a variation of skim milk proteins, human milk amino acid composition also changes during lactation. In the cow and goat milk studies, whey protein: casein ratio was reported as 20:80 (27,28).

Although we focused on the whey fraction and used the ultracentrifugation approach to eliminate casein from colostrum, we still detected casein proteins, especially in GC, in our gels. We believe that the significant casein fraction in GC hampered the casein elimination in the sample preparation procedures. Still, the rest of the HAPs and LAPs in whey fraction provided a preliminary but important proteomic comparison between the goat and human colostrum.

Conclusions

The whey proteins of human and goat colostrum were a remarkable number of common proteins. Additionally, the enrichment of disease-related pathways in human colostrum suggested a role for passive immunization which might protect the newborn from diseases. Further research in terms of potential allergic responses is needed to better understand whether goat colostrum can be used as an alternative formula for scarce human colostrum in the future.

Statements and Declarations

Declarations of Interest

The authors declare no conflict of interest.

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Ethical Approval

The study was approved by the Acibadem University Ethical Committee (ATADEK-2013-507)

Consent to Participate

Human colostrum samples were collected from subjects who agreed to sign an informed consent form.

Consent to Publish

All authors consent to publish.

Authors Contributions

CAL: Investigation, Resources, Writing-original draft; YU: Formal analysis, Writing-original draft, Writing-review and editing, Visualization; SP: Methodology, Investigation, Writing-review and editing; EU: Formal analysis, Writing-review and editing; US: Formal Analysis, Writing-review and editing; PA: Resources, Writing-review and editing; PAU: Resources, Writing-review and editing; AO: Conceptualization, Supervision, Writing-review and editing, Project administration

Availability of data and materials

All relevant data are within the paper.

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