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Insight into the Effects of In Vitro-Digested Donkey and Sheep Milk on Differentiated Human Intestinal Caco-2 Cells

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Abstract

The chemical composition of donkey and sheep milk showed significant differences, with donkey milk containing less fat and protein and a higher level of lactose, while sheep milk had more dry matter and a higher proportion of casein and saturated, unsaturated, monounsaturated, and polyunsaturated fatty acids. This study investigated the biological effects of in vitro-digested donkey and sheep milk, and their blends at different ratios, on differentiated human intestinal Caco-2 cells. Gene expression analysis showed that treatment with digested donkey milk (100%) significantly altered the expression of the autophagy-related gene (*SQSTM1*), tight junction (*CLDN4*), and innate defense genes (*DEFB1*, *MUC2*, and *MUC5*), showing a non-cytotoxic response and a profile consistent with a barrier-related transcriptional response. In contrast, sheep milk (100%) and certain milk blends (70% and 50% sheep's milk) down-regulated genes involved in the autophagy process (*ULK1*, *AMBRA*, *BECN1*, *ATG5*, *GABARAP*, and *SQSTM1*), tight junction genes (*OCLN* and *CDH1*), and innate defense genes (*DEFB1* and *MUC2*), suggesting a distinct epithelial response that warrants further confirmation. Metabolomic profiling revealed clear compositional and functional distinctions among the digested milks, identifying 166 metabolites that were differentially regulated (136 compounds were down-regulated and 30 up-regulated). Donkey milk digestion yielded a metabolite profile enriched in polar peptides and compounds with reported antioxidant associations, whereas sheep milk digestion showed a different pattern dominated by hydrophobic peptides and lipid-related metabolites. These findings indicate that, under the tested in vitro conditions, donkey milk digestion is associated with gene expression and metabolomic patterns consistent with epithelial barrier support.

Keywords: donkey milk; sheep milk; donkey–sheep milk blend; Caco-2 cell; gene expression; metabolomics

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1. Introduction

Milk remains an important component of human diets worldwide because of its nutrient and bioactive compounds, including proteins, peptides, lipids, oligosaccharides,

vitamins, and minerals [1]. Beyond its traditional dietary role, milk is increasingly studied for its potential health-related functions [2]. Recently, consumer demand for traditional, healthy, and distinctive products has led to a significant increase in the production of milk and milk products from autochthonous mammalian species [3,4]. Due to its health benefits, milk from non-bovine species is considered an alternative, nutritious source [5]. According to OECD-FAO forecasts, global milk production should reach 1146 million tons by 2034, with the strongest growth in regions with currently lower yields, such as India, Pakistan, and countries in sub-Saharan Africa [6]. While cow's milk remains the dominant source, milk from other species accounts for nearly 20% of total output. Milk from minor species, such as donkeys, represents a negligible fraction of global volume, but it is increasingly recognized for its distinct functional and nutritional properties [5,7]. Donkey milk is rich in bioactive proteins, essential fatty acids, and a favorable amino acid profile, which confer antimicrobial, anti-inflammatory, and immunomodulatory effects, making it an appealing alternative for nutritionally sensitive populations [8,9]. In addition to the nutritional components of milk, biologically active peptides (BAPs) produced by digestion with gastrointestinal enzymes play a key role in the functionality of milk [10]. Milk-derived peptides exhibit antioxidant activity in Caco-2 cells by activating the Keap1/Nrf2 pathway, which leads to the overexpression of antioxidant enzymes [11]. Also, donkey milk significantly ameliorates clinical symptoms and reduces histopathological damage in the colon in mice with induced ulcerative colitis. Furthermore, it might promote intestinal repair in ulcerative colitis by suppressing the TLR4/MyD88/NF-κB signaling pathway [12]. From a technological perspective, donkey milk presents challenges due to its relatively low fat and protein content, particularly its low casein-to-whey protein ratio, which limits its suitability for traditional milk processing, such as cheese production [13,14]. In addition, its high price and low yield pose additional challenges in dairy product production. A promising strategy is combining donkey milk with casein-rich milks, such as sheep milk, to create blends that balance biological benefits with improved technological performance [15]. Sheep milk, characterized by its high total solids and casein content, offers excellent processing properties, particularly in cheese and fermented dairy production [16,17]. This approach may help generate dairy products that combine technological suitability with distinct biological properties. Beyond traditional nutritional aspects, recent research has focused on milk-derived components and their potential health benefits, especially for gastrointestinal health [18].

To our knowledge, this is the first study to evaluate digested donkey and sheep milk, alone and in combinations, in a differentiated human Caco-2 cell model alongside untargeted metabolomic profiling. We hypothesized that digested donkey milk would induce a distinct transcriptional and metabolomic response compared with sheep milk due to its unique compositional characteristics. This study aimed to compare the cellular and metabolomic responses elicited by simulated digestion products of donkey milk, sheep milk, and their blends in different ratios.

2. Materials and Methods

2.1. Sample Collection

Milk samples were collected in April 2024 from lactating animals of two autochthonous breeds: Balkan donkeys (n = 35) and the White Metohija strain of Pramenka sheep (n = 30), differing in age and lactation stage, during a single-batch milking session for each species. All sampled animals were in good physical condition, clinically healthy, and without visible signs of disease or abnormalities in feeding or behavior. Sampling was carried out within the area of the "Stara Planina" Nature Park, a protected natural asset of exceptional importance (Category I) in Eastern Serbia. Before milking, the udder

was washed with warm water without disinfectants and dried using disposable paper towels. Milk samples were collected aseptically into sterile containers with dark bottoms during hand milking, after visual examination of the foremilk for abnormalities (e.g., clots, flakes, or discoloration), and after discarding the first streams of milk. Manual milking was performed by the farm owner using disposable gloves, which were changed between each animal. Immediately after collection, the samples were labeled and placed in a portable refrigerator maintained at approximately 4 °C, where they remained under refrigerated conditions throughout transport to the laboratory. Upon arrival at the laboratory, individual donkey and sheep milk samples were separately pooled, homogenized, and subdivided into three equal portions to generate composite samples which served as a technical replicate, thereby simulating the practical pooling of milk from a larger number of animals in routine milk collection. Subsequently, the composite donkey and sheep milk samples were then used to independently prepare technical replicates (n = 3) of five donkey–sheep milk blends containing 100%, 70%, 50%, 30%, or 0% donkey milk (v/v), with the remaining volume consisting of sheep milk, were prepared before further analyses. All procedures involving animals were conducted in accordance with the Serbian Law on Animal Welfare. The study protocol was approved by the Ministry of Agriculture, Forestry, and Water Management of the Republic of Serbia (Approval No. 002787498 2026/1).

2.2. Raw Milk Analysis

Raw milk analyses were performed using a FOSS CombiFoss™ FT+ system, which combines the MilkoScan™ FT+ and Fossomatic™ FC modules. The proximate composition (fat, saturated, monounsaturated, and polyunsaturated fatty acids, protein, lactose, total solids, and solids-not-fat) was determined by mid-infrared spectrometry according to ISO 9622:2020 [19], while somatic cell counts were determined by flow cytometry according to ISO 13366-2:2008 [20]. For raw milk analysis, the pooled donkey and sheep milk samples were homogenized and analyzed in three technical replicates (n = 3).

2.3. In Vitro Digestion of Milk Samples

In vitro gastrointestinal digestion was performed following the standardized static in vitro digestion protocol [21]. Milk blends containing 100%, 70%, 50%, 30%, and 0% donkey milk were prepared by independently mixing the appropriate volumes of pooled donkey and sheep milk. Each blend was prepared in three independent ways (technical replicates, n = 3), and each replicate was subjected to the in vitro gastrointestinal digestion procedure separately. Briefly, 22.5 mL of each milk sample was mixed with 15.75 mL of Simulated Salivary Fluid (SSF; 0.5 mol/L KCl (Sigma-Aldrich, St. Louis, MO, USA), 0.5 mol/L KH_2PO_4 (Serva Electrophoresis GmbH, Heidelberg, Germany), 1 mol/L $NaHCO_3$ (Serva), 2 mol/L NaCl (Biolife Italiana, Milan, Italy), 0.15 mol/L $MgCl_2(H_2O)_6$ (Sigma-Aldrich), 0.5 mol/L $(NH_4)_2CO_3$ (Sigma-Aldrich), pH = 7). α-amylase (Sigma-Aldrich) was resuspended in 2.25 mL SSF and added to achieve a final activity of 75 U/mL. $CaCl_2$ (Sigma-Aldrich) was added to the mixture to obtain a final concentration of 0.75 mM. Then, 4.5 mL of water was added to reach a final mixture volume of 45 mL. The mixture was incubated at 37 °C for 2 min with shaking at 180 rpm. Then, 45 mL of oral-phase mixture was then mixed with 41 mL of Simulated Gastric Fluid (SGF; 15.1 mol/L KCl, 3.7 mol/L KH_2PO_4, 6.8 mol/L $NaHCO_3$, 0.15 mol/L $MgCl_2(H_2O)_6$, 0.5 mol/L $(NH_4)_2CO_3$, pH = 3), with the addition of pepsin (Sigma-Aldrich) at 2000 U/mL activity. Water was added to reach a final volume of 90 mL, and $CaCl_2$ was added to 0.075 mM. The pH of the final gastric mixture was adjusted to 3 with 3 M HCl (PanReac AppliChem, Darmstadt, Germany), and the mixture was incubated at 37 °C for 120 min with constant shaking at 180 rpm to simulate peristaltic movements. The resulting gastric mixture was then mixed with

72 mL of Simulated Intestinal Fluid (SIF; 6.9 mol/L KCl, 0.9 mol/L KH_2PO_4, 25 mol/L $NaHCO_3$, 47.2 mol/L NaCl, 0.1 mol/L $MgCl_2(H_2O)_6$, 0.5 mol/L $(NH_4)_2CO_3$, pH = 7), with added bovine bile salts (Fluka, Buchs, Switzerland) at a concentration of 4.7 mg/mL. Pancreatin (Sigma-Aldrich) was added to achieve a protease activity of 800 U/mL in the intestinal-phase mixture. Water was added until the volume reached 180 mL, and the pH was adjusted to 7.0 with 3 M NaOH (Supelco, Bellefonte, PA, USA). The intestinal-phase mixture was incubated at 37 °C for 120 min with constant shaking at 180 rpm. After digestion, the samples were frozen at –20 °C. To prepare an intestinal juice control, digestive fluids, salts, and enzymes were mixed in the same proportions as those used during the in vitro digestion procedure, but without the addition of milk. This control was used to evaluate the contribution of the digestion reagents alone. Following in vitro digestion, the total protein concentrations of the digested milk samples were determined using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions.

2.4. Cell Culture and Treatments

Differentiated human Caco-2 cells were used as an in vitro model of the small intestinal epithelial barrier, as previously described [22]. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, 100 U/mL penicillin, and 2 mM L-glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). They were maintained in 75 cm^2 flasks at 37 °C in a humidified atmosphere containing 5% CO_2. Caco-2 cells were seeded in a 24-well plate (2×10^5 cells/well) and incubated at 37 °C for 21 days to allow differentiation, resulting in a total of 2×10^6 cells/well. After differentiation, cells were treated for 4 h with in vitro-digested milk samples (milk blends containing 100%, 70%, 50%, 30% and 0% donkey milk, including intestinal juice control) normalized to a final protein concentration of 100 µg/mL. The concentration was selected based on preliminary dose-finding experiments showing no detectable cytotoxicity. Untreated Caco-2 cells cultured in complete medium without the addition of digested samples served as the negative control. In parallel, differentiated Caco-2 cells were treated with the intestinal juice control (intestinal juice) to assess the effect of the digestion reagents alone. Following treatment, the cells were washed three times with PBS to remove excess product, followed by a trypsinization step to detach the cells (Trypsin-EDTA, Torlak, Belgrade, Serbia).

2.5. Cytotoxicity Assay

A lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to evaluate the cytotoxicity of in vitro-digested milk applied to Caco-2 cells. After 4 h of treatment, supernatants were collected, and released LDH was detected according to the manufacturer's instructions. The absorbance was measured at 490 nm using the Infinite 200 Pro Plate Reader (MTX Lab Systems, Männedorf, Switzerland).

2.6. RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from Caco-2 cells using Trizol reagent (Thermo Fisher Scientific) according to [23]. Isolated RNA was treated with DNase I (RapidOut DNA Removal Kit) according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA) to remove potential contamination with genomic DNA. RevertAid Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), random hexamers (Thermo Fisher Scientific, Waltham, MA, USA), and RiboLock RNase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA) were used for the transcription of 1 µg of isolated RNA. Quantitative real-time PCR (qPCR) was performed under the following conditions: 2 min at 95 °C activation, 40 cycles of 5 s at 95 °C and 30 s at 60 °C in Line-Gene 9600 Plus Real-Time

PCR (Hangzhou Bioer Technology, Hangzhou, Zhejiang, China) by using IC Green qPCR Universal Kit (NIPPON Genetics, Düren, Germany). Normalization was performed against the *GAPDH* gene (Ct values for *GAPDH* were compared across different treatments and independent experiments, confirming that *GAPDH* expression remained stable under the experimental conditions used), using the $2^{-\Delta\Delta Ct}$ method. Primers of the genes used in the study are listed in Table 1.

Table 1. List of the primers used in this study.

Gene Name	Primer Sequence 5′–3′	Reference
*GAPDH*_F	GTGAAGGTCGGAGTCAACG	[24]
*GAPDH*_R	TGAGGTCAATGAAGGGGTC	
*ULK1*_F	TTTTGTTTCTCCGTTGGGGC	[25]
*ULK1*_R	ACTCTTCCCGGGCTGCTAAT	
*AMBRA1*_F	GGTGGGAGGAGAGGGGATAG	[25]
*AMBRA1*_R	CGAGGGGCATGTCATCATTT	
*BECN1*_F	CTGGGACAACAAGTTTGACCAT	[26]
*BECN1*_R	GCTCCTCAGAGTTAAACTGGGTT	
*ATG5*_F	CACAAGCAACTCTGGATGGGATTG	[27]
*ATG5*_R	GCAGCCACGGACGAAACAG	
*MAP1LC3B*_F	TTCAGGTTCACAAAACCCGC	[25]
*MAP1LC3B*_R	TCTCACACAGCCCGTTTACC	
*GABARAP*_F	CCCTCGTCCCGCTGATTTTA	[25]
*GABARAP*_R	ATCCCTCCAGCTTGTACCCA	
*SQSTM1*_F	GCCAGAGGAACAGATGGAGT	[28]
*SQSTM1*_R	TCCGATTCTG GCATCTGTAG	
*CLDN4*_F	ACAGACAAGCCTTACTCC	[29]
*CLDN4*_R	GGAAGAACAAAGCAGAG	
*OCLN*_F	TCAGGGAATATCCACCTATCACTTCAG	[29]
*OCLN*_R	CATCAGCAGCAGCCATGTACTCTTCAC	
*CDH1*_F	AGCCTGTCGAAGCAGGATTG	[25]
*CDH1*_R	AGAAACAGCAAGAGCAGCAGA	
*DEFB1*_F	TGTCTGAGATGGCCTCAGGT	[30]
*DEFB1*_R	GGGCAGGCAGAATAGAGACA	
*MUC2*_F	CACCTGTGCCCTGGAAGGC	[31]
*MUC2*_R	CGGTCACGTGGGGCAGGTTC	
*MUC5*_F	CGGGTCCACGAGGAGACGGT	[31]
*MUC5*_R	GCTTCTGCAGCCAGGCACGA	

2.7. Extraction Protocol for Metabolomic Analysis of Digested Donkey and Sheep Milk

The different samples were extracted according to the protocol of [32]. Briefly, in vitro-digested milk blends containing 100%, 70%, 50%, 30% and 0% donkey milk, including intestinal juice control (2 mL), were diluted to 4 mL acetonitrile LC–MS grade, acidified with 3% formic acid, vortexed for 15 min, and centrifuged at 5000 *g* for 10 min at 4 °C to remove the precipitate. One mL of the supernatant was then filtered in a vial through a 0.45 µm cellulose syringe filter before HPLC–HRMS analysis, by injecting 5 µL of the extract. Three procedural blank samples (without matrix) were extracted under the same conditions as the digested milk blends and intestinal juice samples under investigation to obtain the exclusion list. In contrast, all the extracted samples were pooled by mixing equal aliquots to make a pool and obtain a quality control sample.

2.8. HPLC–HRMS Analysis of Digested Donkey and Sheep Milk

Chromatographical separation was carried out on Vanquish HPLC system (Thermo Fisher Scientific, San Jose, CA, USA), using an Accucore C18 column (100 × 2 mm i.d., 4 µm, (4 × 3 mm i.d.) (Thermo Fisher Scientific, San Jose, CA, USA) and the mobile phase

consisted of water (A) and methanol (B) both acidified with 0.1% formic acid. An Orbitrap Exploris 120 (Thermo Scientific, San Jose, CA, USA) was used as a detector. All the chromatographic conditions and HRMS parameters are reported in the work of [32]. AcquireX software v3.0 (Thermo Scientific, San Jose, CA, USA) with the Deep Scan workflow was used to create a background ion exclusion list from the procedural blank and an ion inclusion list from the sample pool (quality control) with dynamic modification of these inclusion and exclusion lists between each replicate injection. An untargeted metabolomics approach with the Compound Discoverer™ workflow, Compound Discoverer (CD) 3.4 program, elaborated the raw data from Orbitrap Exploris 120 analysis for chemical identification and statistical evaluation.

2.9. Statistical Analysis

The results are presented as mean values ± standard deviation (SD). All experiments were repeated at least three times independently, and each set of experiments was performed in triplicate. For gene expression analyses, each treatment group was compared with the untreated control using unpaired two-tailed Student's t-tests. To account for multiple testing across genes and treatment comparisons, *p*-values were adjusted using the Benjamini–Hochberg false discovery rate procedure in GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). Adjusted p-values < 0.05 were considered statistically significant. Statistical analyses and graph preparation were conducted using GraphPad Prism 10 unless otherwise stated. For metabolomic analysis, descriptive, univariate, and multivariate statistics were performed using the Differential Analysis node within the Compound Discoverer workflow, including PCA, HCA, volcano plots, and box-and-whisker charts. For multigroup comparisons, one-way ANOVA followed by Tukey's post hoc test was applied, and *p*-values were adjusted using the Benjamini–Hochberg algorithm.

3. Results

3.1. Proximate Chemical Composition of Raw Milk

The proximate composition and selected quality characteristics of the pooled donkey and sheep milk samples used in this study are presented in Table 2. The values represent the mean ± SD of technical replicate measurements and are intended to characterize the experimental material.

Table 2. Proximate composition and selected physicochemical characteristics of pooled samples of donkey and sheep milk.

	Donkey Milk	**Sheep Milk**
Fat (%)	0.43 ± 0.1	7.45 ± 0.2
Protein (%)	1.32 ± 0.1	6.97 ± 0.15
Lactose (%)	6.01 ± 0.21	3.14 ± 0.1
Fat-free dry matter (%)	7.86 ± 0.1	11.29 ± 0.15
Dry matter (%)	8.29 ± 0.15	18.74 ± 0.2
Freezing point (m °C)	−466 ± 1	−524 ± 1
Somatic cells × 1000/mL)	13 ± 4	247 ± 6
Urea (mg/dL)	19.7 ± 0.1	34.8 ± 0.1
Casein (%)	1.06 ± 0.05	5.16 ± 0.1
Saturated fatty acids (%)	0.309 ± 0.02	4.552 ± 0.1
Unsaturated fatty acids (%)	0.063 ± 0.005	2.456 ± 0.02
Monounsaturated fatty acids (%)	0.173 ± 0.002	2.333 ± 0.005
Polyunsaturated fatty acids (%)	0.211 ± 0.01	0.531 ± 0.01

Note: Values are presented as the mean ± SD of technical replicate measurements performed on pooled milk samples. No inferential statistical comparisons were performed because the measurements represent technical replicates of a single pooled sample for each milk type.

Donkey milk contained lower fat (0.43%) and protein (1.32%) contents than sheep milk (7.45% and 6.97%, respectively), whereas its lactose content was higher (6.01% vs. 3.14%). The urea concentration was 19.7 mg/dL in donkey milk and 34.8 mg/dL in sheep milk, while the casein content was 1.06% and 5.16%, respectively. Sheep milk also exhibited higher saturated (4.552%) and unsaturated fatty acid contents (2.456%) than donkey milk (0.309% and 0.063%, respectively). Although donkey milk had a lower total fat content, it contained a relatively higher proportion of polyunsaturated fatty acids. Additionally, the FOSS CombiFoss™ FT+ instrument employs calibration models primarily developed for bovine milk, whereas the present study included donkey milk, which differs substantially in chemical composition and fatty acid profile. Consequently, the estimated fatty acid fractions for donkey milk should be interpreted as instrument-derived estimates rather than absolute quantitative values. Sheep milk showed higher fat-free dry matter (11.29%) and total dry matter (18.74%) than donkey milk (7.86% and 8.29%, respectively). The freezing point was −466 m °C for donkey milk and −524 m °C for sheep milk, while the somatic cell count was 13 × 10^3/mL and 247 × 10^3/mL, respectively.

3.2. Gene Expression in Differentiated Caco-2 Cells

The next goal of this study was to examine the effects of in vitro-digested donkey milk, sheep milk, and blends prepared at different donkey milk–sheep milk ratios on differentiated human intestinal Caco-2 cells. To assess whether autophagy-related pathways could be modulated by the digested milk samples, we performed qPCR analysis of genes involved in different stages of the autophagy process. For most autophagy-related genes, significantly lower mRNA levels were observed in cells treated with in vitro-digested sheep milk (0:100) and with the 50:50 and 30:70 donkey–sheep milk ratios compared with the negative control (Figure 1). In contrast, *SQSTM1* expression is significantly higher in cells treated with in vitro-digested donkey milk.

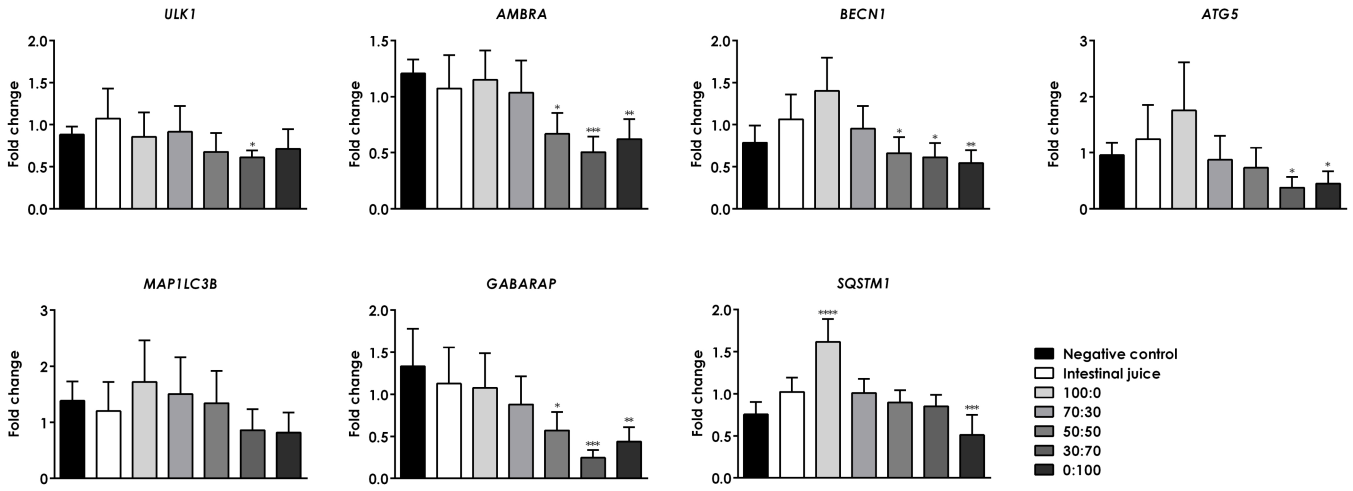


Figure 1. Expression of autophagy-related genes in differentiated Caco-2 cells treated with in vitro-digested donkey milk (100:0), sheep milk (0:100), and donkey–sheep milk blends (70:30, 50:50, and 30:70). Numbers in parentheses indicate the percentage of each milk type used. Data are presented as mean values ± SD. Statistical significance was assessed using unpaired two-tailed Student's *t*-tests with Benjamini–Hochberg correction for multiple comparisons; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

We further analyzed the expression of genes associated with epithelial junctions. In cells treated with in vitro-digested donkey milk, *CLDN4* expression was significantly increased, while *OCLN* and *CDH1* expression were significantly decreased in cells treated with in vitro-digested sheep milk (0:100) and in those treated with the 30:70 donkey–sheep

milk blend (Figure 2A). We also examined the expression of genes encoding human β-defensin and mucins. The results showed increased *DEFB1*, *MUC2*, and *MUC5* mRNA levels after treatment with in vitro-digested donkey milk (100:0) compared with the control (Figure 2B). In contrast, significant decreases in *DEFB1* and *MUC2* expression were observed after treatment with in vitro-digested sheep milk, as well as with the 30:70 donkey–sheep milk blend. The intestinal juice control and all digested milk samples were compared with the negative control (untreated Caco-2 cells) to assess their effects on gene expression. The results showed that intestinal juice alone did not significantly alter the expression of the investigated genes compared with the negative control. Therefore, the observed biological effects were attributed to the digested milk blends rather than components from the simulated intestinal digestion medium.

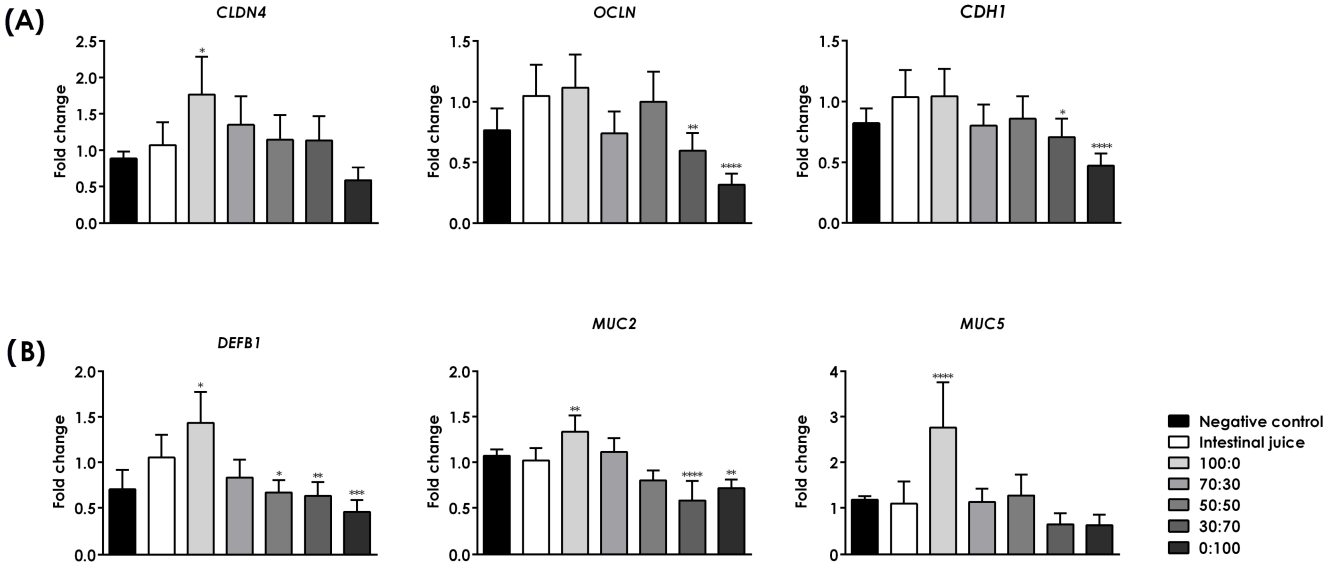


Figure 2. Expression of tight junction protein genes (**A**) and human β-defensin and mucin genes (**B**) in differentiated Caco-2 cells treated with in vitro-digested donkey milk (100:0), sheep milk (0:100), and donkey–sheep milk blends (70:30, 50:50, and 30:70). Numbers in parentheses indicate the percentage of each milk type used. Data are presented as mean values ± SD. Statistical significance was assessed using unpaired two-tailed Student's *t*-tests with Benjamini–Hochberg correction for multiple comparisons; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

To ensure that the observed modulation of autophagy-related pathways was not due to cytotoxic effects induced by the in vitro-digested milk samples, which could trigger autophagy as a cell death mechanism, LDH release in the culture medium of treated Caco-2 cells was measured. No significant differences in LDH release were observed between treated cells and the negative control (untreated Caco-2 cells) (Figure 3), indicating that the tested samples did not induce detectable cytotoxicity under the applied experimental conditions. Cell viability was expressed relative to the negative control, which was set at 100%. Although some treatment groups exhibited mean viability values slightly below the control, these differences were not statistically significant and fell within the expected experimental variability of the LDH assay. Therefore, neither the in vitro-digested milk samples nor their blends reduced Caco-2 cell viability under the experimental conditions.

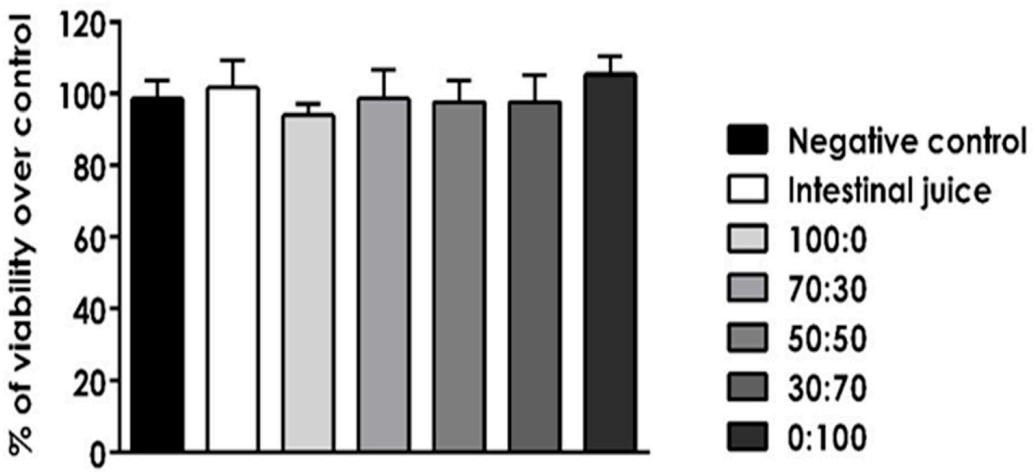


Figure 3. Effect of in vitro-digested donkey (100:0) and sheep (0:100) milk, as well as blends of donkey and sheep milk (ratios of these milks 70:30, 50:50, and 30:70) on Caco-2 cell viability measured by lactate dehydrogenase (LDH) assay. Numbers in parentheses indicate the percentage of each type of milk used.

3.3. Metabolomics Analysis of In Vitro-Digested Milk Samples

Considering the different responses in Caco-2 cells elicited by the tested milks, metabolomics analysis of the in vitro-digested samples was subsequently performed to elucidate potential underlying biochemical mechanisms. To obtain a more reliable number of metabolites in a complex matrix, the metabolomics investigation in this study was performed in both positive and negative ionization modes in separate analytical sessions. Overall, 366 of 9062 items were confirmed in the positive electrospray ionization mode (Table S1).

The initial overall statistical visualization is reported in Figure 4, which shows the PCA score plot of ESI+, based on the confirmed metabolites. The plot is based on the first 2 principal components (PC) for the acquired data, explaining 78% of the total variance in the dataset of ESI+ elaboration, with six visible clusters for the different digested milk compositions. In particular, the digested milks consisting of either 100% donkey milk (100:0) or 100% sheep milk (0:100) are found in the opposite quadrants; in the center is the 50:50 composition, with the 70:30 and 30:70 donkey: sheep compositions on the sides. The clear clusterization was also confirmed in the heat map reported in Figure 5 for the ESI+ data elaboration.

Regarding differential analysis, several markers (up- and down-regulated compounds) discriminating the different compositions of digested milk have emerged. In particular, 136 compounds were down-regulated (p value ≤ 0.05, $\log_2$ fold change ≤ -2) and 30 up-regulated (p value ≤ 0.05, $\log_2$ fold change ≥ 2) in 100:0 digested donkey–sheep milk compared with the 0:100 donkey–sheep blend (Table S2, Figure 6) after ESI+ data processing.

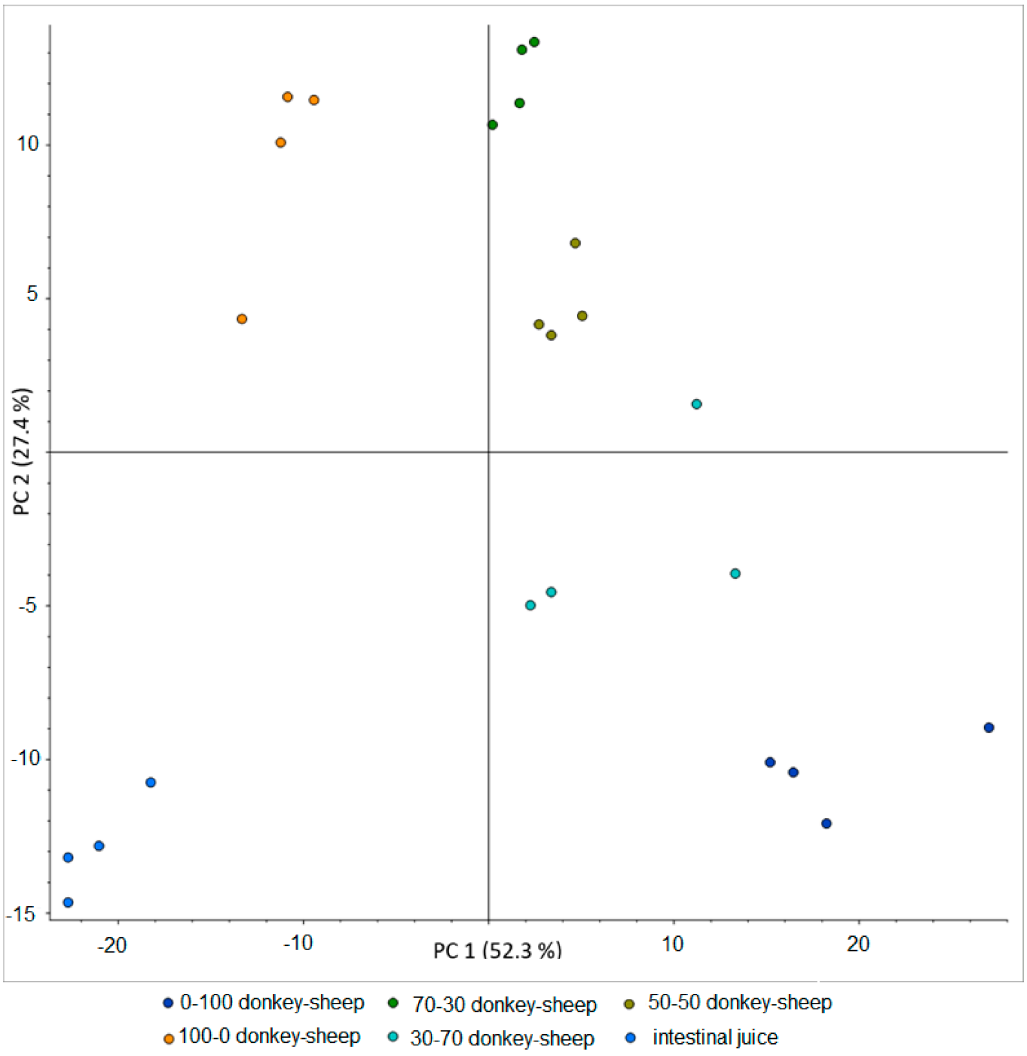


Figure 4. PCA score plot of ESI+ data.

Furthermore, overall, 285 of 8493 items were confirmed in the negative electrospray ionization mode (Table S3). The PCA score plot is also based on the first 2 principal components (PCs) of the acquired data, explaining the same percentage (78%) of the total variance for the ESI− elaboration dataset. The same arrangement of the 6 clusters is also observed in the PCA of the ESI data (Figure 7), following a logic of proximity between the groups that is effectively congruent with the different composition percentages and is further confirmed by the heat map (Figure 8).

As regards differential analysis, several markers (up- and down-regulated compounds) discriminating the different compositions of digested milk have also emerged after ESI− data elaboration. In particular, 91 compounds were down-regulated and 24 up-regulated in 100–0 digested donkey–sheep milk in comparison with 0–100 donkey–sheep (Table S4, Figure 9) after ESI− data elaboration.

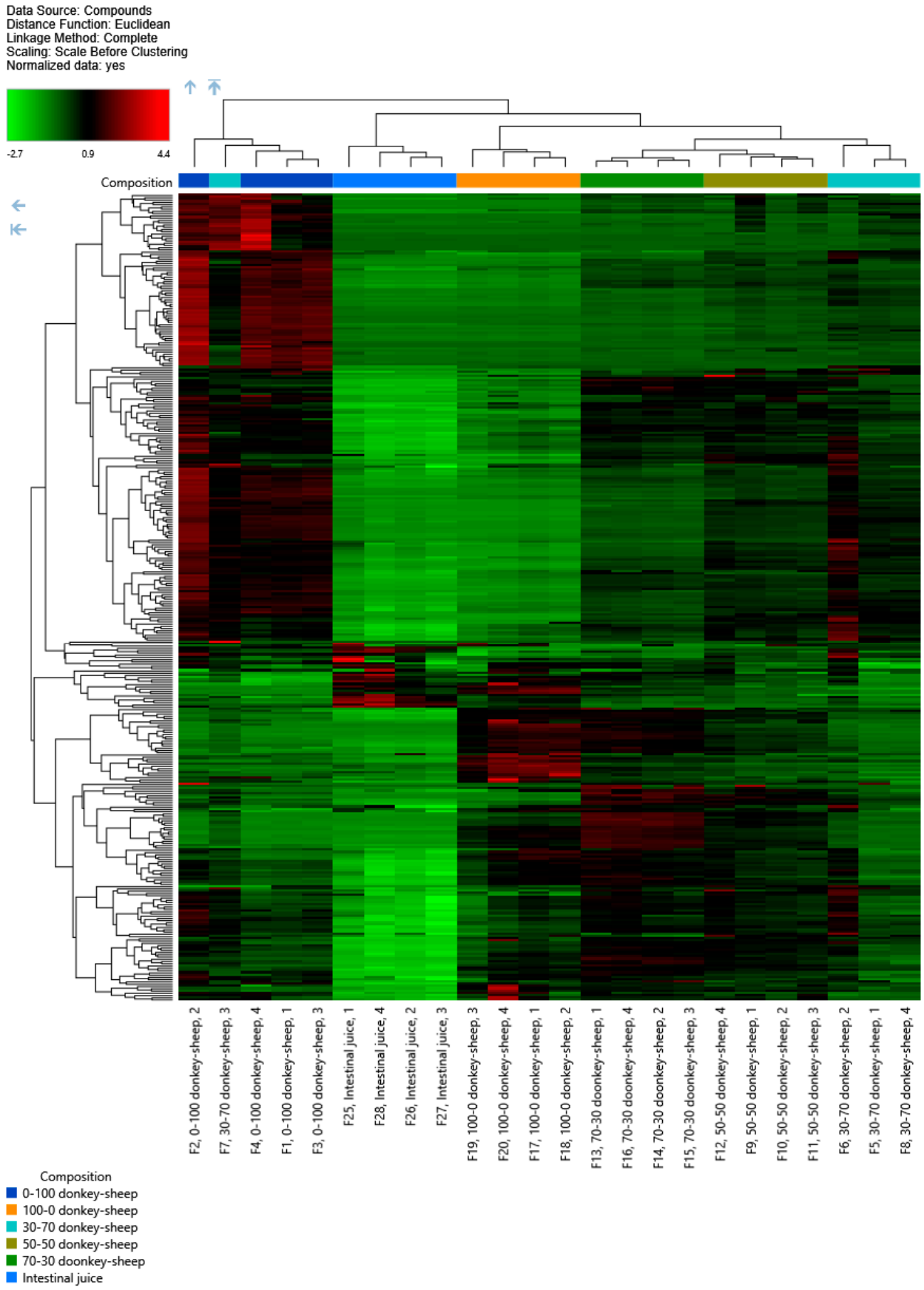


Figure 5. Hierarchical Cluster Analysis with heat map of ESI+ data elaboration.

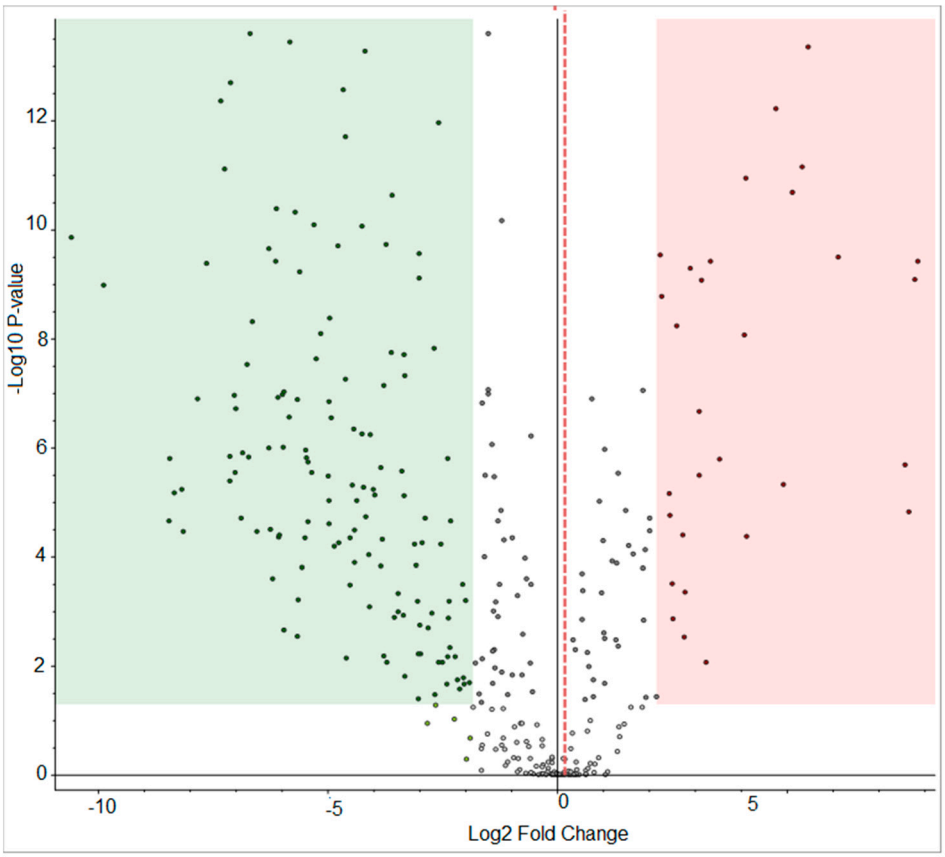


Figure 6. Volcano plot of the comparison 100–0 donkey–sheep vs. 0–100 donkey–sheep in ESI+ data elaboration.

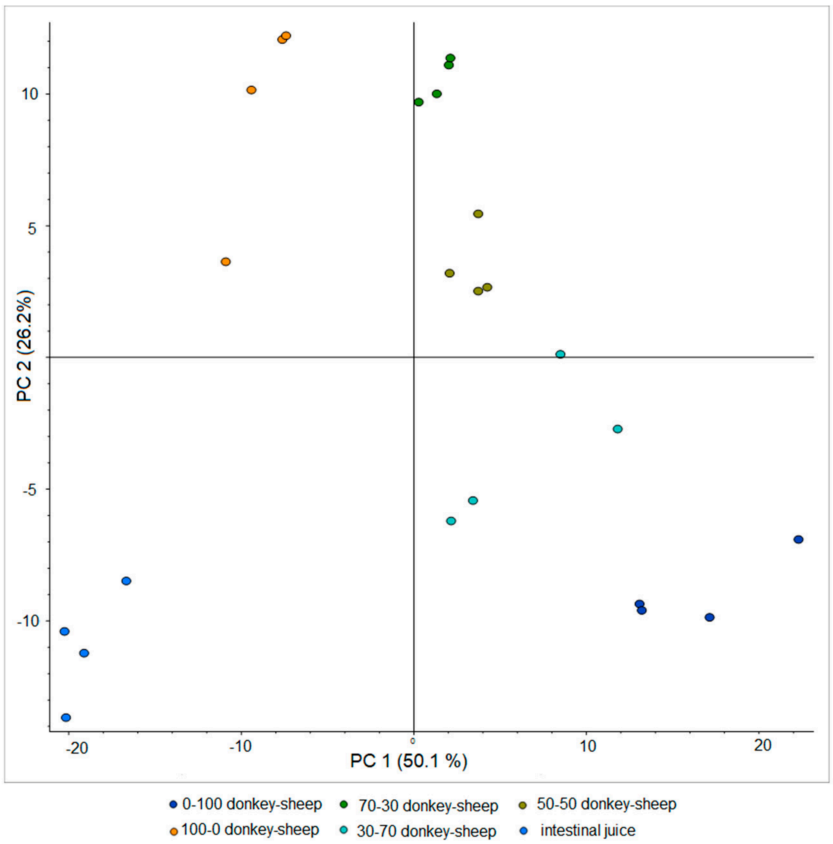


Figure 7. PCA score plot of ESI− data.

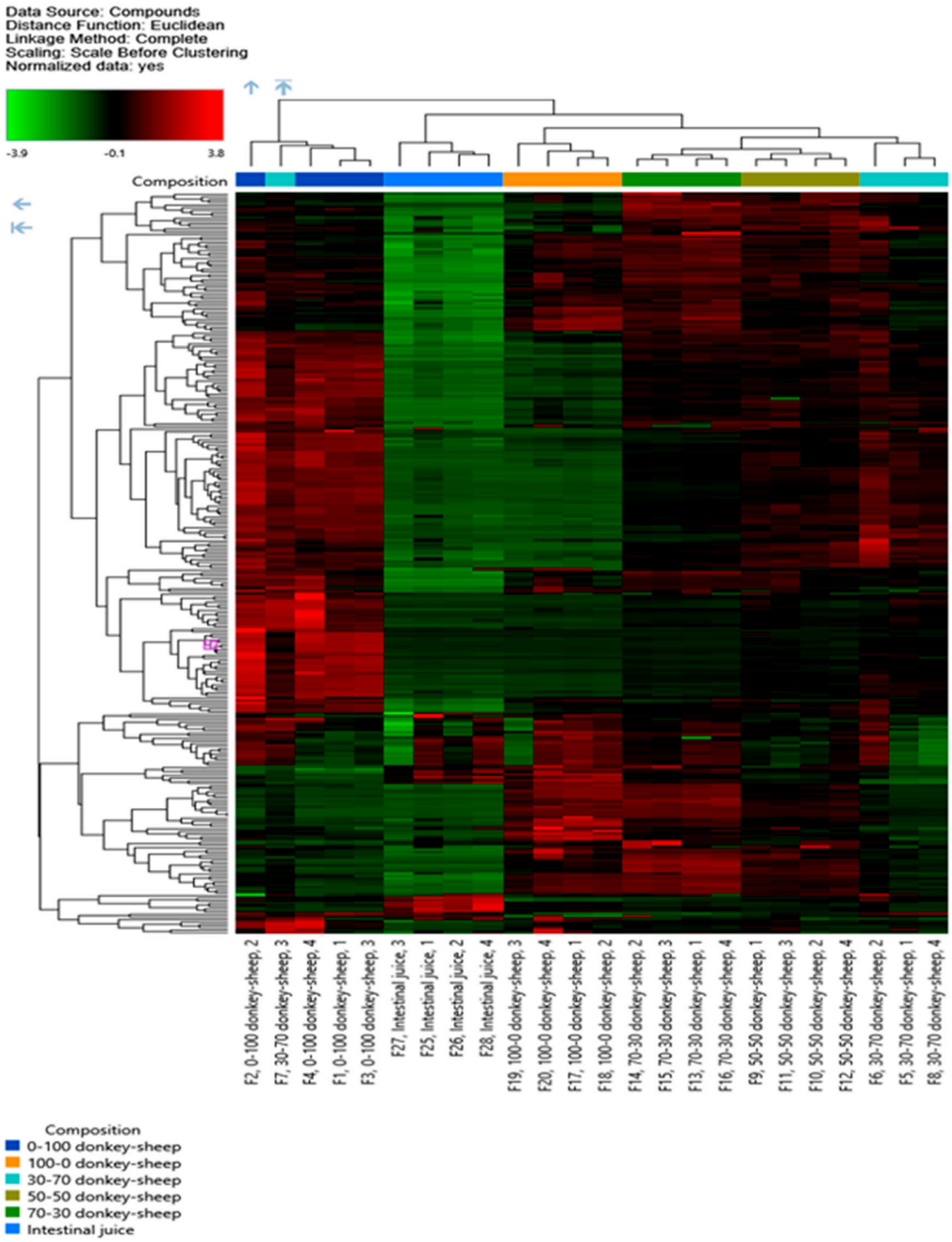


Figure 8. Hierarchical Cluster Analysis with heat map of ESI− data elaboration.

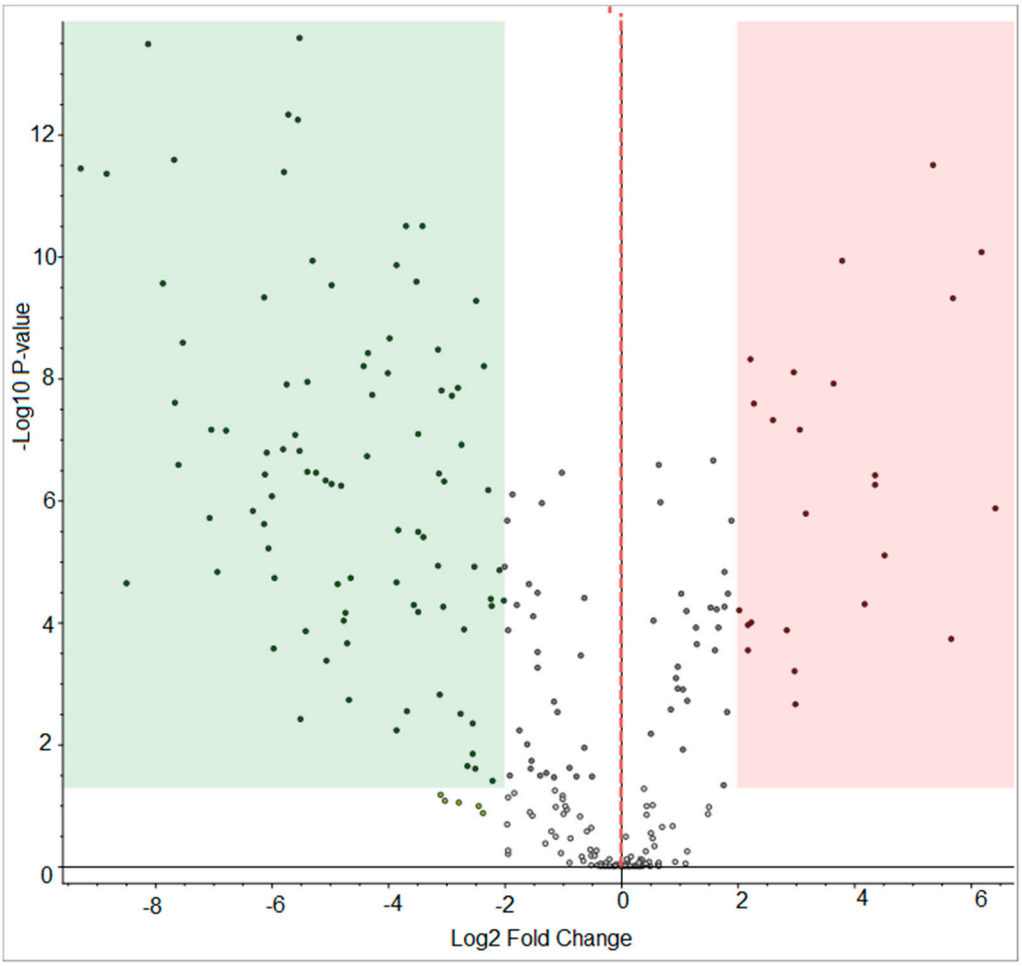


Figure 9. Volcano plot of the comparison 100–0 donkey–sheep vs. 0–100 donkey–sheep in ESI− data elaboration.

4. Discussion

Chemical analysis of raw milk revealed differences in the proximate composition of donkey and sheep milk samples. Donkey milk showed lower fat (0.43%) and protein (1.32%) contents compared with sheep milk (7.45% and 6.97%, respectively), whereas a higher lactose content was observed in donkey milk (6.01%) compared with sheep milk (3.14%). The higher lactose concentration contributes to the characteristic sweet taste of donkey milk and has been reported as one of the features that makes its composition more comparable to human milk [33,34]. Sheep milk exhibited higher total dry matter and non-fat dry matter values, reflecting its greater concentration of milk solids and supporting its suitability for dairy processing applications [16]. The somatic cell count values were lower in donkey milk (13×10^3/mL) compared with sheep milk (247×10^3/mL). Although somatic cell count is commonly used as an indicator of milk quality, these values should be interpreted in the context of species-specific characteristics and management conditions [34,35]. The casein content was lower in donkey milk (1.06%) than in sheep milk (5.16%), which is consistent with previous reports describing donkey milk as an albuminous milk with a lower casein proportion and weaker coagulation properties, limiting its suitability for traditional cheesemaking [14,36]. Sheep milk showed higher saturated (4.55%) and unsaturated fatty acid contents (2.46%) compared with donkey milk. Although donkey milk has a lower total fat content, its fatty acid profile includes a relatively higher proportion of polyunsaturated fatty acids, which may contribute to its nutritional value [37]. However, limitations due to FTIR calibration models that were

primarily developed for bovine milk should be considered when interpreting the fatty acid profile of donkey milk, as species-specific calibration would be required for more accurate absolute quantification. Due to its higher content of fat and protein, sheep milk is commonly considered more suitable for the production of cheese and fermented dairy products [38]. In contrast, donkey milk is characterized by a lighter structure, higher lactose content, and a protein profile with a higher proportion of whey proteins, which has been associated with its digestibility and potential suitability as an alternative milk source for some nutritionally sensitive populations [34]. Overall, the observed compositional differences between donkey and sheep milk reflect their distinct nutritional and technological characteristics and provide the basis for their different applications in food and nutrition.

Considering the characteristics of both milks, we further examined the effects of in vitro-digested samples on differentiated Caco-2 cells. Autophagy is an important cellular process involved in intestinal physiology, including the maintenance of cellular homeostasis and the regulation of epithelial function [39,40]. Because only transcriptional changes were assessed, the present findings should be interpreted as changes in the expression of autophagy-related genes rather than direct evidence of autophagy activation. In this context, our results are consistent with previous studies showing that food-derived components may modulate autophagy-related pathways in mammalian cells [41,42]. As autophagy has been linked with the improvement of tight junction barrier function in Caco-2 cells [42], the up-regulation of tight junction proteins correlates with the induction of autophagy, pointing to a strong role of autophagy in controlling paracellular TJ permeability by targeting pore-forming tight junction protein claudin-2 [43,44]. Finally, the important feature of functional foods is the potential to stimulate production of antimicrobial peptides (e.g., defensins) and mucins, which play important roles in host defense [45,46]. After non-cytotoxic treatment of Caco-2 cells with in vitro-digested donkey milk (100:0), increased expression of *SQSTM1*, *CLDN4*, *DEFB1*, *MUC2*, and *MUC5* was observed, suggesting modulation of autophagy-related, epithelial barrier-related, and innate defense-related pathways. Although changes in tight junction gene expression suggest potential modulation of epithelial barrier function, functional validation using TEER measurements or paracellular permeability assays was not performed and should be addressed in future studies. In contrast, exposure to in vitro-digested sheep milk (0:100) led to a down-regulation of all examined genes involved in autophagy, intercellular junctions, defensins, and mucins, suggesting a gene expression pattern that differs from that elicited by donkey milk and may reflect a lesser barrier-associated effect at the transcriptional level.

Metabolomic profiling revealed both shared and divergent features between the down- and up-regulated compounds identified after in vitro digestion. In both groups, the majority of metabolites consisted of small peptides (di- and tripeptides), reflecting extensive proteolytic activity during digestion [47]. Several amino acid derivatives and lipid-related metabolites were also present in both datasets, including monoacylglycerols and medium-chain fatty acids, indicating that protein and lipid metabolism were commonly affected. However, clear compositional differences were evident between the two profiles. Down-regulated compounds identified by differential analysis included metabolites belonging to different chemical classes, including peptides and lipid-related molecules (Table S2). Some of these metabolites have previously been associated with pathways involved in inflammatory regulation. In contrast, up-regulated metabolites were mainly polar peptides containing acidic or aromatic residues (aspartic acid, glutamic acid, tyrosine, threonine) and conjugated amino acid derivatives such as hippuric acid, compounds previously associated with antioxidant or protective functions. Moreover, the accumulation of glycerol monoesters and sorbitan derivatives among the up-regulated compounds suggests enhanced lipid remodeling or re-esterification processes. The overall metabolic pattern indicates

substantial compositional differences between the digested milk samples. Although several identified metabolites have been previously associated with inflammatory or antioxidant processes, the present untargeted metabolomics approach does not allow direct inference regarding pathway activation or suppression. These findings are consistent with the cellular responses observed after treatment with in vitro-digested donkey milk, supporting the view that donkey milk digestion generates a distinct bioactive profile relative to sheep milk, although confirmation in additional models is needed.

5. Conclusions

This study demonstrates that donkey and sheep milk differ not only in composition but also in the bioactive signals generated after simulated digestion. In differentiated Caco-2 cells, digested donkey milk elicited an autophagy-related and epithelial barrier-associated transcriptional profile, together with increased expression of genes involved in mucosal defense, whereas digested sheep milk induced a different response. Untargeted metabolomics further confirmed that digestion of the two milk types yields distinct molecular signatures, supporting the view that milk species origin shapes post-digestive bioactivity. The study is limited by its in vitro design, the use of a single cell line, and the absence of protein-level and functional validation. Taken together, these findings position donkey milk as a distinct dairy matrix with the potential to generate digestion-derived signals relevant to intestinal barrier homeostasis and mucosal defense.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/dairy7040059/s1: Table S1. Items were confirmed in the positive electrospray ionisation mode; Table S2. Down- and up-regulated compounds in the positive electrospray ionisation mode in 100–0 donkey-sheep digested milk in comparison with the 0–100 donkey-sheep digested milk; Table S3. Items were confirmed in the negative electrospray ionisation mode; Table S4. Down- and up-regulated compounds in the negative electrospray ionisation mode in 100–0 donkey-sheep digested milk in comparison with the 0–100 donkey-sheep digested milk.

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Data Availability Statement: The original contributions presented in this study are included in the Supplementary Materials. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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