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The Role of Milk Exosomal microRNAs in Balancing the Nutrition between Buffalo Breeds with Low and High Milk Production

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ABSTRACT

Background: Milk is the primary nutrition source for mammalian infants. Production of milk varies among species, breeds within the same species or even individuals of the same breed. Despite large variations in milk production (*e.g.* yield, composition), the health of recipient infants remains unperturbed regardless of species, breeds or individuals, suggesting a compensatory mechanism for low milk production to offset the potential nutrition difference compared with high milk production.

Methods: Here, we profiled and compared the milk exosomal microRNAs between groups with low and high milk production in two buffalo breeds.

Result: We found that individuals with low milk production mainly down-regulated microRNAs targeting the genes in protein digestion and absorption pathway. Given that milk exosomal microRNAs could be taken up by the epithelial cells of infants, down-regulation of which inhibits expression of genes encoding proteins responsible for protein digestion, peptides and amino acid transportation in infants' digestive system. Such down-regulation of genes facilitates the protein digestion, peptide absorption and amino acid absorption (*e.g.* stomach, intestine, colon) for infants. Our results thus provide a novel insight that exosomal microRNAs may play an important role in balancing the nutrient absorption of infants between mothers with low milk production and ones with high milk production.

Key words: Exosomal microRNA, Milk production.

INTRODUCTION

Milk is produced by the mammary glands of mammals and composed of multiple bioactive components, such as proteins, peptides, fatty acids, oligosaccharides, beneficial microorganisms. It is well recognized that milk comprises the primary source of nutrition for infants (Van Winckel *et al.*, 2011). Breastfeeding is the dominant means to feed the milk to infants in almost all mammals. Accumulating studies have demonstrated that breast milk plays an irreplaceable role in compensating for developmental delays in immune function in the neonate and it can also reduce the permeability of the intestine to prepare it for extrauterine life (Goldman, 2000). Besides, breast milk can affect infants' growth, development and self-regulation of food intake since it is enriched with hormones, neuropeptides and growth factors (Savino and Liguori, 2008). As a result, researchers have suggested that breast milk serves as the best nutrition, immune protection and regulation of growth, development and metabolism for infants of mammals (Goldman, 2012).

Interestingly, milk composition and production (normalized by body weight) vary from species to species, even different breeds of the same species (Aqib *et al.*, 2019). For example, the milk yield of the yak cow is much less than that of dairy cattle. Nevertheless, their respective infants don't exhibit significant differences in health conditions. This raises an interesting scientific question: Why could a species (or breed, individual) with lower milk production raise their infants as healthy as those with higher milk production?

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Effective food digestion is essential to guarantee the health of infants. It is well known that milk contains multiple enzymes (amylase and lipase) which aid digestion (Hamosh, 1996). Recently, some research reported that exosomes, a type of extracellular vesicle containing constituents (protein, DNA and RNA) of the cells that secrete them (Kalluri and LeBleu, 2020), can be isolated from both colostrum and mature milk (Admyre *et al.*, 2007). Importantly, it has been

reported that milk exosome microRNAs can be taken up by intestinal epithelial cells and stimulate gastric/pancreatic digestion (Liao *et al.*, 2017). Moreover, Kahn *et al.* found that milk exosomal microRNAs can directly stimulate infant gut digestion (Kahn *et al.*, 2018). These studies highlight that, apart from digestive enzymes, milk exosomes serve an essential role in facilitating food digestion in infants. We thus hypothesize that the property of milk exosomes in individuals with low milk production may be different from those with high milk production, which may contribute to the comparable health conditions of respective infants.

To test this hypothesis, we extracted the exosomes from milk of two buffalo breeds (Murrah and Nili-Ravi buffalos), both of which have individuals with low and high milk production. The microRNAs were profiled to unravel their differential expression (and their target genes) between low and high productive groups, underlying the potential roles of microRNAs in the two groups. This study supplies genetic data to helps improve buffalo molecular breeding.

MATERIALS AND METHODS

All animal procedures (milking) were conducted in accordance with the Animal Care and Use Committee of the Guangxi Buffalo Research Institute, Chinese Academy of Agricultural Sciences. The experimental animals were kept on the farm of Guangxi Buffalo Research Institute, with proper feeding conditions. We selected six healthy Murrah female buffalos (three with low milk production M6629, M333, M4578 and the other three with high milk production M991, M3102, M1190) and six Nili-Ravi buffalos (three with low milk production M4556, M6815, M1975 and the other three with high milk production M6137, M4336, M4516) and milked them by hand. For pasteurization, the milk was heated at 71.6°C for 15 seconds. We chose fat-free pasteurized milk rather than ultra-heat treated milk, as the latter causes a loss of milk microRNAs and presumably exosomes (Kirchner *et al.*, 2016). Exosomes were isolated by ultracentrifugation as described previously (Izumi *et al.*, 2015; Wolf *et al.*, 2015). The combined use of fat-free milk and exosome purification by ultracentrifugation minimized sample contamination with fat globules, which may also contain microRNAs (Alsaweed *et al.*, 2016). Exosomes were authenticated by transmission electron microscopy, nanoparticle tracking analysis (NanoSight NS300, Malvern, Inc.) and western blot analysis following the guidelines provided by the International Society for Extracellular Vesicles (Kusuma *et al.*, 2016; Lötvall *et al.*, 2014). For each exosome, RNA was isolated from milk and milk exosomes following the protocol of treated with TRIzol reagent (ThermoFisher) and RNA with length 18-30nt was recovered by PAGE electrophoresis. cDNA synthesis was performed by using the miScript Reverse Transcription Kit following the manufacturer's instructions (Qiagen), with the products subjected for library construction, followed by sequencing on BGISEQ-500 sequencing platform.

We used SOAPnuke (1.5.0) (Chen *et al.*, 2018) to filter the raw sequencing data. Briefly, we removed the reads: 1) with more than four bases with base quality less than ten or more than six bases with base quality less than 13; 2) contaminated with 5' adaptor sequence; 3) without 3' adaptor sequence; 4) without inserts; 5) with consecutive '*A*' bases longer than 60% of the total length; with length less than 15 nucleotides. Then, we used Bowtie2 (2.2.9) (Langmead *et al.*, 2009) to align the clean data to the reference genome of buffalo with parameters: -q -L 16 -p 6 -phred64 -rdg 1,10 -rfg 1,10. Only the reads with at most one mismatch were retrieved for further analysis.

To carry out microRNA annotation on the retrieved sequences of a sample, we first aligned the mature miRNA sequences of all animals in the miRBase (V22) database (Kozomara *et al.*, 2019) onto the buffalo genome by Bowtie2 (2.2.9) (Langmead and Salzberg, 2012). For those miRNAs that were completely mapped onto the genome, we extracted the aligned regions along with 150bp flanking fragments and fed them into RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) to predict hairpin structure. The ones with a hairpin structure were used as miRNA database of buffalo. Next, we mapped the retrieved reads onto the miRNA database using Bowtie2, with the ones exactly matched considered as miRNAs. As for those retrieved reads that fail to map onto the miRNA database, we further annotated them based on Rfam (V12.1, https://rfam.xfam.org/) database using cmsearch (Nawrocki and Eddy, 2013). Finally, the annotation results were integrated as the final annotated miRNA for the studied sample.

To determine the expression of each miRNA, we calculated the TPM (UMI count of this miRNA × 1000000 / total UMI count) for each miRNA in each sample. We further applied DESeq2 (Love *et al.*, 2014) to conduct differential expression analysis between low milk production and high milk production groups, with corrected *p-value* (*FDR*) ≤0.5 being considered to be significant different.

Next, we used miRanda (3.3a) (John *et al.*, 2004) to predict the target genes for the identified miRNA with parameters set as: -en -20 -strict. We also used RNAhybrid (Kruger and Rehmsmeier, 2006) (parameters: - b 100 -c -f 2,8 -m 100000 -v 3 -u 3 -e -20 -p 0.8) to perform this prediction. The target genes supported by either miRanda or RNAhybrid were kept for further analysis. Finally, we performed functional enrichment analysis based on GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) on these target genes using Hypergeometric test, with the term having a corrected *p-value* (*FDR*) d" 0.05 defined to be significant different.

RESULTS AND DISCUSSION

We generated approximately 25 million clean tags per sample, with over 98% having a Phred score ≥ 20, suggesting the high quality of our sequencing data (Table 1). Annotation of these clean tags identified multiple known miRNA and novel miRNA in these samples, ranging from

Table 1: Summary of sequencing information.

Sample ID	Total raw reads	Total clean reads	Clean reads ratio (%)	Clean reads Q20(%)	Low quality reads ratio (%)	Invalid adapter reads ratio (%)	PolyA reads ratio (%)	Length less 15 nt reads ratio(%)
M1190	32,876,712	23,983,817	72.95	98.90	0.58	7.46	0.01	19.00
M3102	29,166,895	27,780,298	95.25	98.80	0.34	2.84	0.00	1.58
M4578	57,142,857	24,053,222	42.09	98.20	0.20	20.61	0.00	37.10
M6333	51,063,829	24,089,671	47.18	98.90	0.62	21.26	0.01	30.93
M6629	28,379,488	25,670,045	90.45	98.70	0.65	4.25	0.00	4.64
M991	29,375,924	24,263,555	82.60	98.60	0.72	8.20	0.01	8.48
N1975	30,379,746	24,090,406	79.30	98.80	0.30	3.05	0.00	17.35
N4516	29,629,629	24,016,803	81.06	98.70	0.34	5.00	0.00	13.60
N4556	40,000,000	24,028,709	60.07	98.50	0.16	15.88	0.00	23.89
N6137	29,621,134	27,429,534	92.60	98.50	0.15	6.17	0.00	1.08
N6815	29,791,709	27,337,538	91.76	98.60	0.30	2.26	0.00	5.67
N987	30,142,154	28,669,836	95.12	99.00	0.59	2.73	0.01	1.55

437 to 808 and from 44 to 999, respectively. RNAhybrid and miRanda identified a total of 20, 201 target genes for all of these miRNAs. Differential expression analysis on these miRNAs identified 388 miRNA up-regulated and 208 down-regulated in low milk production group relative to high milk production group for Murrah buffalos and 128 up-regulated and 1,106 down-regulated in low milk production group for Nili-Ravi buffalos.

To find out the common features in groups with low milk production, we first extracted the genes significantly up-regulated by miRNA in low milk production groups for both Murrah and Nili-Ravi buffalos. GO enrichment analysis identified ten significantly enriched GO terms, including intrinsic to membrane, integral to membrane, proteinaceous extracellular matrix, plasma membrane, membrane part, cell periphery, membrane, extracellular matrix part, extracellular space, intrinsic to plasma membrane (Table 2). KEGG enrichment analysis detected 12 significantly enriched pathways, including protein digestion and absorption, amoebiasis, AGE-RAGE signaling pathway in diabetic complications, ECM-receptor interaction, PI3K-Akt signaling pathway, platelet activation, focal adhesion, cytokine-cytokine receptor interaction, other types of O-glycan biosynthesis, cocaine addiction, vibrio cholerae infection and small cell lung cancer (Table 3). Protein digestion and absorption pathway exhibited the most significant difference among all pathways (*FDR* = 1.75E-24).

Next, we analyzed the genes significantly down-regulated by miRNA in low milk production groups for both buffalo breeds. These genes were significantly enriched on only two GO terms (plasma membrane, cell periphery) and eight KEGG pathways (Protein digestion and absorption, Amoebiasis, ECM-receptor interaction, Notch signaling pathway, Focal adhesion, AGE-RAGE signaling pathway in diabetic complications, Platelet activation, Insulin secretion) (Table 4). Of particular note is that the pathway showing the most significant difference is still protein digestion and absorption pathway (*FDR* = 9.38E-14), the same as the findings mentioned above (Table 3 and Table 4).

Table 2: GO enrichment for genes targeted by miRNA up-regulated in low milk production groups.

GO term	Function description	Q-value
GO:0031224	Intrinsic to membrane	1.94E-09
GO:0016021	Integral to membrane	4.81E-08
GO:0005578	Proteinaceous extracellular matrix	1.32E-06
GO:0005886	Plasma membrane	1.46E-05
GO:0044425	Membrane part	1.49E-05
GO:0071944	Cell periphery	1.56E-05
GO:0016020	Membrane	0.00715
GO:0044420	Extracellular matrix part	0.01521
GO:0005615	Extracellular space	0.02452
GO:0031226	Intrinsic to plasma membrane	0.04199

We were interested in looking at the protein digestion and absorption pathway in detail. Protein digestion and absorption pathway mainly describe the processes of protein hydrolysis in stomach and absorption of multiple amino acids in the small intestine and distal colon. When mapping all of the genes targeted by down-regulated miRNAs in low milk production groups, we found that these genes cover over 80% of the genes in this pathway (Fig 1). On the other hand, analysis of the genes targeted by up-regulated miRNAs revealed that less than 50% of the genes in the studied pathway were covered (Fig 1). Particularly, all of these genes overlapped with the ones targeted by down-regulated miRNAs (Fig 1). This observation could be explained as these down-regulated or up-regulated miRNAs targeted on different isoforms in the same gene locus. Under such circumstance, the effect on such gene expression was difficult to determine, which warrants further investigation in the future (Fig 1). Since genes with down-regulated miRNAs have a higher coverage on the Protein digestion and absorption pathway compared with those with up-

Table 3: KEGG enrichment for genes targeted by miRNA up-regulated in low milk production groups.

Pathway ID	Pathway	DEGs genes with pathway annotation (6916)	All genes with pathway annotation (17134)	Q-value
ko04974	Protein digestion and absorption	345 (4.99%)	550 (3.21%)	1.749547e-24
ko05146	Amoebiasis	314 (4.54%)	535 (3.12%)	4.996697e-16
ko04933	AGE-RAGE signaling pathway in diabetic complications	213 (3.08%)	372 (2.17%)	2.495909e-09
ko04512	ECM-receptor interaction	196 (2.83%)	341 (1.99%)	7.440679e-09
ko04151	PI3K-Akt signaling pathway	318 (4.6%)	638 (3.72%)	3.263614e-05
ko04611	Platelet activation	203 (2.94%)	386 (2.25%)	3.332727e-05
ko04510	Focal adhesion	264 (3.82%)	521 (3.04%)	4.039443e-05
ko04060	Cytokine-cytokine receptor interaction	132 (1.91%)	244 (1.42%)	3.583483e-04
ko00514	Other types of O-glycan biosynthesis	29 (0.42%)	42 (0.25%)	5.687514e-03
ko05030	Cocaine addiction	41 (0.59%)	67 (0.39%)	1.463216e-02
ko05110	Vibrio cholerae infection	117 (1.69%)	229 (1.34%)	1.796318e-02
ko05222	Small cell lung cancer	89 (1.29%)	170 (0.99%)	2.666499e-02

Table 4: KEGG enrichment for genes targeted by miRNA down-regulated in low milk production groups.

Pathway ID	Pathway	DEGs genes with pathway annotation (12860)	All genes with pathway annotation (17134)	Q-value
ko04974	Protein digestion and absorption	488 (3.79%)	550 (3.21%)	9.379052e-14
ko05146	Amoebiasis	450 (3.5%)	535 (3.12%)	2.871669e-05
ko04512	ECM-receptor interaction	288 (2.24%)	341 (1.99%)	1.579203e-03
ko04330	Notch signaling pathway	79 (0.61%)	86 (0.5%)	4.828284e-03
ko04510	Focal adhesion	427 (3.32%)	521 (3.04%)	5.443691e-03
ko04933	AGE-RAGE signaling pathway in diabetic complications	309 (2.4%)	372 (2.17%)	6.189917e-03
ko04611	Platelet activation	317 (2.47%)	386 (2.25%)	2.089203e-02
ko04911	Insulin secretion	110 (0.86%)	126 (0.74%)	2.089203e-02

regulated miRNAs and considering the unknown effects on genes targeted by both down- and up-regulated miRNAs, we focused on the genes targeted by exclusively down-regulated miRNAs in this pathway. In this study, we identified nine such genes in this pathway (Fig 1).

Given that miRNA usually represses the expression of its target gene (Cannell *et al.*, 2008), the down-regulation of miRNA implies that the suppression effects on the expression of the target gene are relieved. The expression of the gene regulated by down-regulated miRNA should be higher than that regulated by up-regulated miRNA. One of the genes regulated exclusively by down-regulated miRNA is a gene encoding Pepsin, expressed in the gastric chief cells of the stomach lining. Pepsin is the principal acid protease of the stomach, serving to break down proteins into smaller peptides (Tang and Lin, 2004). Another noteworthy gene down-regulated by miRNA is *SLC15A1* (Solute Carrier Family 15 Member 1), highly expressed in the small intestine and encoding a protein that mediates the uptake of small peptides from the lumen into the enterocytes (Frazier *et al.*, 2008). It has been reported that exosomal microRNAs in milk from mothers can be taken up by infants' (*e.g.* intestinal cells) (Liao *et al.*, 2017), implying that exosomal microRNA can potentially regulate the expression of genes from infants. In this respect, individuals with low milk production may down-regulated these milk exosomal microRNAs, which can relieve the suppression on the expression of pepsin-encoding gene and *SLC15A1* in their infants to enhance the efficiency of protein digestion and peptide absorption.

Apart from the two abovementioned genes, *SLC1A1* (Solute Carrier Family 1 Member 1), which encodes a member of the high-affinity glutamate transporters that play an essential role in transporting glutamate across plasma membranes (Arnold *et al.*, 2006), was found to be exclusively regulated by down-regulated microRNAs. Glutamate is an important neurotransmitter present in over 90% of all brain synapses in the central nervous system, thus representing the most abundant excitatory neurotransmitter in the vertebrate nervous system (Meldrum, 2000). Moreover, glutamate is also involved in cognitive functions such as learning and memory in the brain (McEntee and Crook, 1993). From this perspective, the down-regulated microRNAs targeting *SLC1A1* could increase the expression level of *SLC1A1* to increase the glutamate transport efficiency, thus maintaining the development of the nervous system in infants.

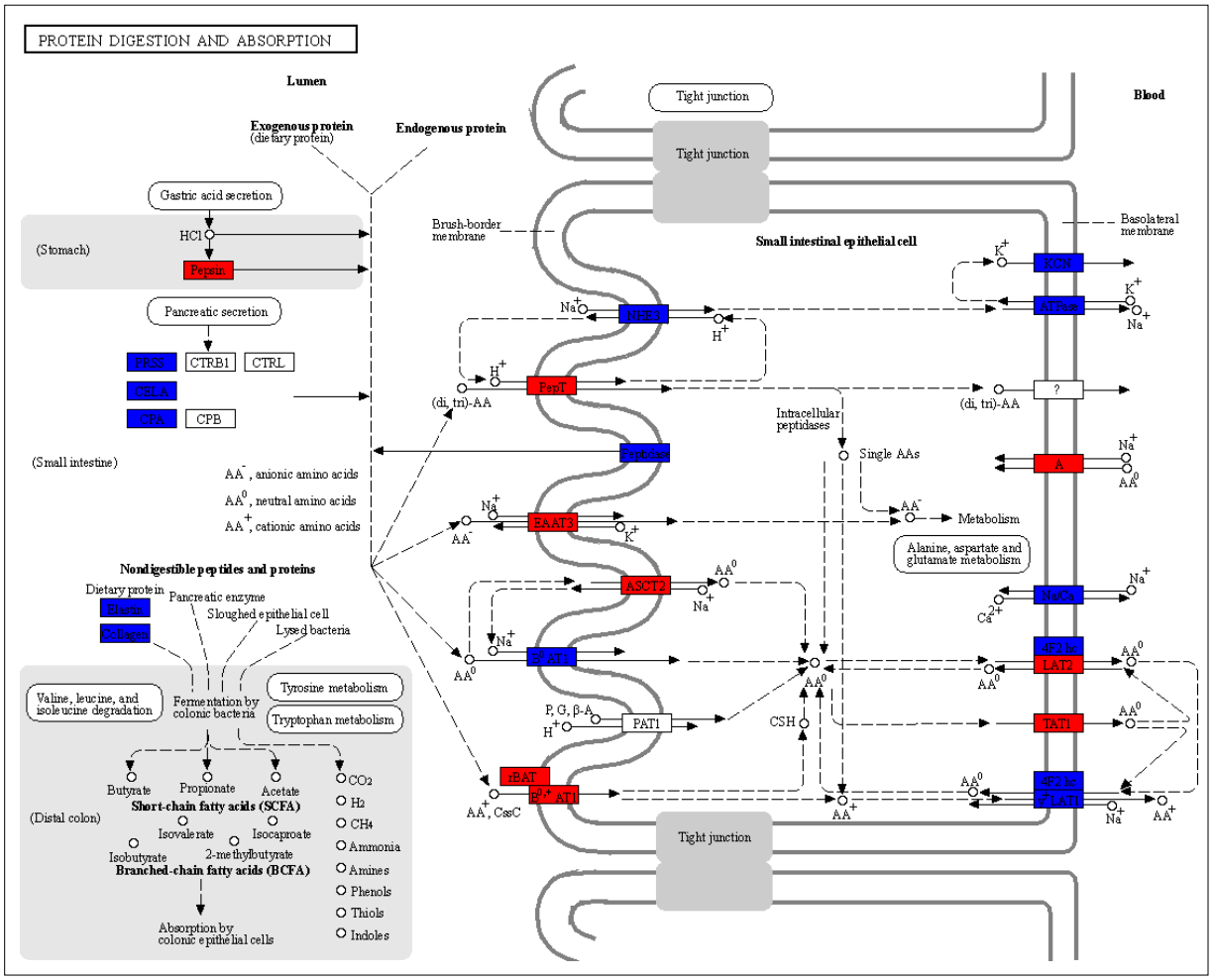

Fig 1: Genes targeted by up-regulated and downregulated miRNA in the Protein digestion and Absorption pathway. Red denotes genes targeted downregulated miRNA and blue denotes genes targeted both downregulated and up-regulated miRNAs.

Interestingly, we found that genes encoding transporters of neutral and basic amino acids were included in the gene set in which the genes were regulated by microRNAs down-regulated in low productive milk. For example, the *SLC1A5* (*Solute Carrier Family 1 Member 5*) gene encodes a sodium-dependent amino acids transporter that accepts all neutral amino acids, including glutamine, asparagine branched-chain and aromatic amino acids as substrates (Garaeva *et al.*, 2018; Kekuda *et al.*, 1996). The *SLC38A2* (*Solute Carrier Family 38 Member 2*) gene, encoding a sodium-dependent amino acid transporter, mediates Na+-coupled cellular uptake of small neutral α-amino acids (AAs) and is extensively regulated in response to humoral and nutritional cues with a stoichiometry of 1:1 (Hoffmann *et al.*, 2018). It may function in the transport of amino acids at the blood-brain barrier. The *SLC7A8* (*Solute Carrier Family 7 Member 8*) gene encodes a sodium-independent, high-affinity transport, with small and large neutral amino acids such as alanine, serine, threonine, cysteine, phenylalanine, tyrosine, leucine, arginine and tryptophan as substrates (Zaragozá, 2020). The *SLC3A1* (*Solute Carrier Family 3 Member 1*) gene encodes a type II membrane glycoprotein, one of the components of the renal amino acid transporter, transporting neutral and basic amino acids in the renal tubule and intestinal tract (Kahya *et al.*, 2021). Conclusively, individuals with low milk production may down-regulate the expression of microRNAs targeting genes encoding transporters for neutral and basic amino acids for better absorption of amino acids in the offspring.

CONCLUSION

In conclusion, buffalo individuals compensate low milk production with upregulation genes involving in the protein digestion and absorption pathway through downregulation miRNA production and consequently increase the efficiency of protein digestion and absorption of amino acids in infants.

Conflicts of interest: None.

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REFERENCES

Admyre, C., Johansson, S.M., Qazi, K.R., Filén, J.J., Lahesmaa, R., Norman, M., Neve, E.P., Scheynius, A., Gabrielsson, S. (2007). Exosomes with immune modulatory features are present in human breast milk. J. Immunol. 179(3): 1969-78.

Alsaweed, M., Lai, C.T., Hartmann, P.E., Geddes, D.T., Kakulas, F. (2016). Human milk miRNAs primarily originate from the mammary gland resulting in unique miRNA profiles of fractionated milk. Sci Rep. 6(1): 20680.

Aqib, A., Ijaz, M., Anjum, A., Kulyar, M.F., Shoaib, M., Farooqi, S. (2019). Reconnoitering Milk Constituents of Different Species, Probing and Soliciting Factors to Its Soundness.

Arnold, P.D., Sicard, T., Burroughs, E., Richter, M.A., Kennedy, J.L. (2006). Glutamate transporter gene SLC1A1 associated with obsessive-compulsive disorder. Arch Gen Psychiatry. 63(7): 769.

Cannell, I.G., Kong, Y.W., Bushell, M. (2008). How do microRNAs regulate gene expression? Biochem Soc Trans. 36: 1224-31.

Chen, Y., Chen, Y., Shi, C., Huang, Z., Zhang, Y., Li, S., Li, Y., Ye, J., Yu, C., Li, Z., Zhang, X., Wang, J., Yang, H., Fang, L., Chen, Q. (2018). SOAPnuke: a MapReduce acceleration-supported software for integrated quality control and preprocessing of high-throughput sequencing data. Gigascience. 7(1): 1-6.

Frazier, S., Ajiboye, K., Olds, A., Wyatt, T., Luetkemeier, E.S., Wong, E.A. (2008). Functional Characterization of the Chicken Peptide Transporter 1 (Pept1, Slc15a1) Gene. Animal Biotechnology. 19(4): 201–10.

Garaeva, A.A., Oostergetel, G.T., Gati, C., Guskov, A., Paulino, C., Slotboom, D.J. (2018). Cryo-EM structure of the human neutral amino acid transporter ASCT2. Nat Struct Mol Biol. 25(6): 515-21.

Goldman, A.S. (2012). Evolution of Immune Functions of the Mammary Gland and Protection of the Infant. Breastfeeding Medicine. 7(3): 132-42.

Goldman, A.S. (2000). Modulation of the Gastrointestinal Tract of Infants by Human Milk. Interfaces and Interactions. An Evolutionary Perspective. The Journal of Nutrition. 130(2): 426S-431S.

Hamosh, M. (1996). Digestion in the newborn. Clin Perinatol. 23(2): 191-209.

Hoffmann, T.M., Cwiklinski, E., Shah, D.S., Stretton, C., Hyde, R., Taylor, P.M., Hundal, H.S. (2018). Effects of Sodium and Amino Acid Substrate Availability upon the Expression and Stability of the SNAT2 (SLC38A2) Amino Acid Transporter. Front Pharmacol. 9: 63.

Izumi, H., Tsuda, M., Sato, Y., Kosaka, N., Ochiya, T., Iwamoto, H., Namba, K., Takeda, Y. (2015). Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages. J Dairy Sci. 98(5): 2920-33.

John, B., Enright, A.J., Aravin, A., Tuschl, T., Sander, C., Marks. D.S. (2004). Human MicroRNA Targets. James C. Carrington, editor. PLoS Biol. 2(11): e363.

Kahn, S., Liao, Y., Du, X., Xu, W., Li, J., Lönnerdal, B. (2018). Exosomal MicroRNAs in milk from mothers delivering preterm Infants survive in vitro digestion and are taken up by human intestinal cells. Mol. Nutr. Food Res. 62(11): 1701050.

Kahya, U., Köseer, A.S., Dubrovska, A. (2021). Amino Acid Transporters on the Guard of Cell Genome and Epigenome. Cancers. 13(1): 125.

Kalluri, R., LeBleu, V.S. (2020). The biology, function and biomedical applications of exosomes. Science. 367(6478): eaau6977.

Kekuda, R., Prasad, P.D., Fei, Y.J., Torres-Zamorano, V., Sinha, S., Yang-Feng, T.L., Leibach FH, Ganapathy V. (1996). Cloning of the sodium-dependent, broad-scope, neutral amino acid transporter Bo from a human placental choriocarcinoma cell line. J. Biol Chem. 271(31): 18657-61.

Kirchner, B., Pfaffl, M.W., Dumpler, J., von Mutius, E., Ege, M.J. (2016). microRNA in native and processed cow's milk and its implication for the farm milk effect on asthma. Journal of Allergy and Clinical Immunology. 137(6):1893-1895.e13.

Kozomara, A., Birgaoanu, M., Griffiths-Jones, S. (2019). miRBase: from microRNA sequences to function. Nucleic Acids Research. 47(D1): D155-62.

Kruger, J., Rehmsmeier, M. (2006). RNAhybrid: microRNA target prediction easy, fast and flexible. Nucleic Acids Research. 34: W451-4.

Kusuma, R.J., Manca, S., Friemel, T., Sukreet, S., Nguyen, C., Zempleni, J. (2016). Human vascular endothelial cells transport foreign exosomes from cow's milk by endocytosis. American Journal of Physiology-Cell Physiology. 310(10): C800-7.

Langmead, B., Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat Methods. 9(4): 357-9.

Langmead, B., Trapnell, C., Pop, M., Salzberg, S.L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10(3): R25.

Liao, Y., Du, X., Li, J,, Lönnerdal, B. (2017). Human milk exosomes and their microRNAs survive digestion *in vitro* and are taken up by human intestinal cells. Mol Nutr Food Res. 61(11): 1700082.

Lötvall, J., Hill, A.F., Hochberg, F., Buzás, E.I., Di Vizio, D., Gardiner, C., Gho, Y.S., Kurochkin, I.V., Mathivanan, S., Quesenberry, P., Sahoo, S., Tahara, H., Wauben, M.H., Witwer, K.W., Théry, C. (2014). Minimal experimental requirements for definition of extracellular vesicles and their functions: A position statement from the International Society for Extracellular Vesicles. J Extracell Vesicles. 3: 26913.

Love, M.I., Huber, W. anders S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15(12):550.

McEntee, W.J., Crook, T.H. (1993). Glutamate: its role in learning, memory and the aging brain. Psychopharmacology. 111(4): 391–401.

Meldrum, B.S. (2000). Glutamate as a Neurotransmitter in the Brain: Review of Physiology and Pathology. The Journal of Nutrition. 130(4):1007S-1015S.

Nawrocki, E.P., Eddy, S.R. (2013). Infernal 1.1: 100-fold faster RNA homology searches. Bioinformatics. 29(22): 2933–5.

Savino, F., Liguori, S.A. (2008). Update on breast milk hormones: Leptin, ghrelin and adiponectin. Clinical Nutrition. 27(1): 42-7.

Tang, J., Lin, X. (2004). Handbook of Proteolytic Enzymes (second edition). Vol. 1. Elsevier.

Van Winckel, M., Vande Velde, S., De Bruyne, R., Van Biervliet, S. (2011). Clinical practice: Vegetarian infant and child nutrition. Eur J Pediatr. 170(12): 1489–94.

Wolf, T., Baier, S.R., Zempleni, J. (2015). The Intestinal Transport of Bovine Milk Exosomes Is Mediated by Endocytosis in Human Colon Carcinoma Caco-2 Cells and Rat Small Intestinal IEC-6 Cells. The Journal of Nutrition. 145(10): 2201-6.

Zaragozá, R. (2020). Transport of Amino Acids Across the Blood-Brain Barrier. Front Physiology. 11: 973.