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C. Boby[1], A. Delavaud[1], J. Pires[1], L.E. Monfoulet[2], S. Bes[1], S. Emery[1], L. Bernard[1], C. Leroux[1], A. Imbert[1], M. Tourret[1], F. Fournier[3], D. Roux[3], H. Sauerwein[4] and M. Bonnet[1]

[1]Université Clermont Auvergne, INRAE, VetAgro Sup, UMR Herbivores, Centre INRAE CARA, 63122 Saint-Genès-Champanelle, France, [2]Université Clermont Auvergne, INRAE, Human Nutrition Unit, Centre INRAE CARA, 63122 Saint-Genès-Champanelle, France, [3]INRAE, Herbipôle, Centre INRAE CARA, 63122 Saint-Genès-Champanelle, France, [4]University of Bonn, Institute of Animal Science, Physiology Unit, Katzenburgweg 7, 53115 Bonn, Germany; celine.boby@inrae.fr

Small extracellular vesicles (EV) are secreted into the extracellular space by all cells. Due to the diversity of their cellular origin and the molecules they contain, small EVs have the ability to ensure extracellular communication and carry molecular signatures of their tissue of origin and its physiological state. In ruminants, early lactation is characterized by profound changes in energy balance and metabolic status. To explore physiological adaptations during early lactation, this study investigated the use of non-invasive milk EVs to identify specific indicators of inter-organ signalling. Milk samples were collected from 8 cows on weeks 2 and 7 postpartum, corresponding to negative and neutral energy balance, respectively. Small EVs were isolated by ultracentrifugation coupled with size exclusion chromatography and characterized by morphological, biophysical and biochemical criteria. Labelled-free shotgun quantitative proteomics was performed by nanoLC-MS/MS. Electron microscopy revealed cup-shaped vesicles with a diameter of about 100 nm, characteristic of small EVs. The diameter was confirmed by Tunable Resistive Pulse Sensing and the specificity of small EVs isolation by the presence of cytoplasmic (TSG101) and membrane (CD63) markers. A total of 508 proteins were identified in milk EVs at weeks 2 and 7 of lactation. Multilevel PCA analysis showed a clear separation between the 2 time points, indicating a strong effect of lactation stage on the protein composition of milk EVs. This proteomic dataset will be analysed to identify small EV molecular signatures of tissue interactions that coordinate nutrient partitioning and adaptation during early lactation.