

Fig. S1 Location of the Fluff layer and casein pellet of ultracentrifuged human and bovine skim milk

Human (left) and bovine (right) skim milk was ultracentrifuged in 39 mL tubes at 340,000 g for 1 hour at 4°C.

The tubes were imaged right before the yellowish Fluff layer was drawn from the tubes.

Approximately 0.5 mL of human Fluff layer (F) and 1 mL of bovine Fluff layer could be drawn using a long glass pipet from one tube of ultracentrifuged skim milk. The marked difference in the casein content of human and bovine milk is reflected in the differently sized casein pellets (C).

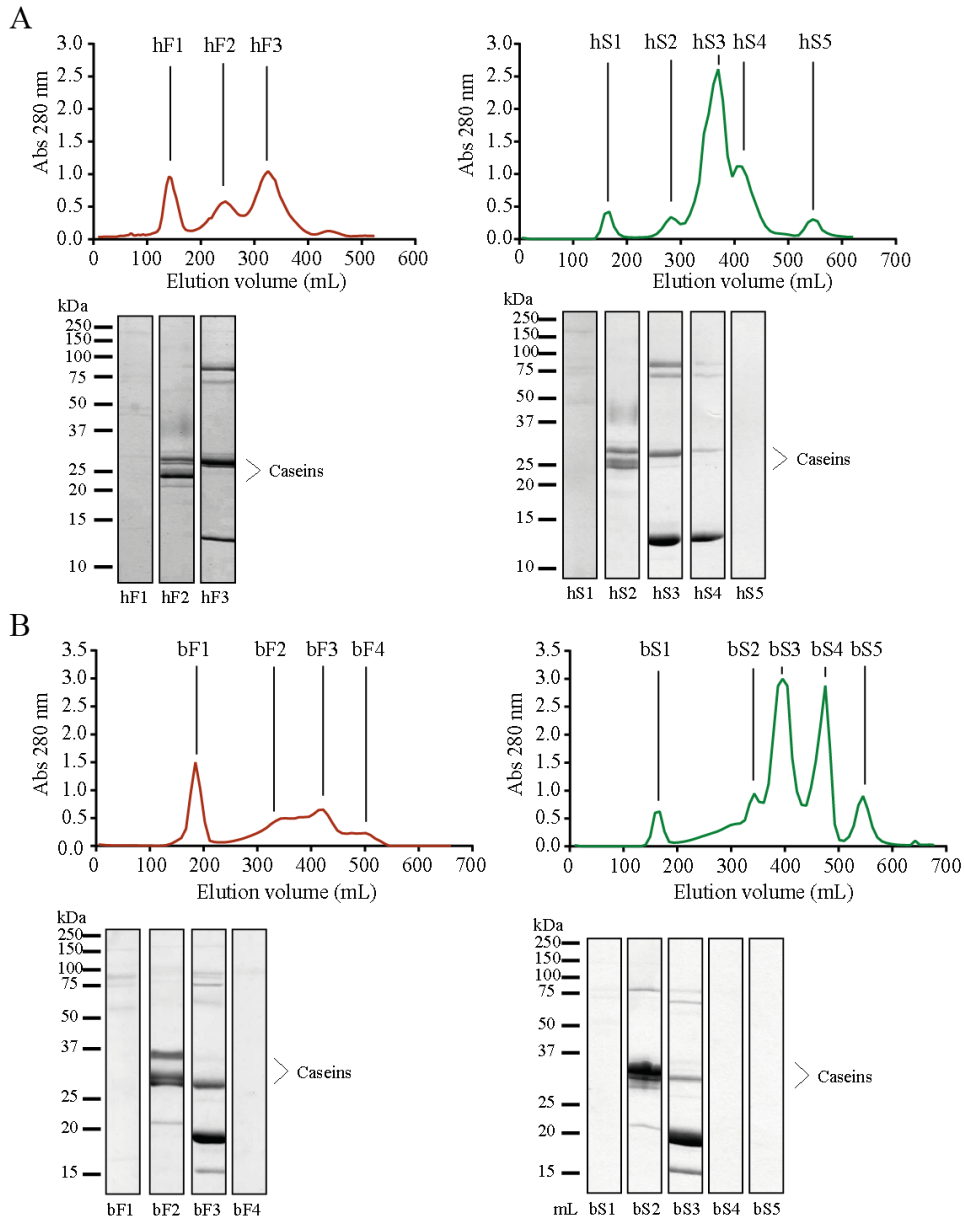


Fig. S2 Protein profiles in Fluff layer and Milk Serum SEC fractions

MEV isolation was performed as described in the materials and methods section.

SEC (size-exclusion chromatography) fractions were normalised to similar 280 nm absorbance values and separated by SDS-PAGE under reduced conditions followed by coomassie blue staining to visualise the fractions protein content.

A. Human Fluff (hF) and Serum (hS) SEC and SDS-PAGE profiles

B. Bovine Fluff (bF) and Serum (bS) SEC and SDS-PAGE profiles.

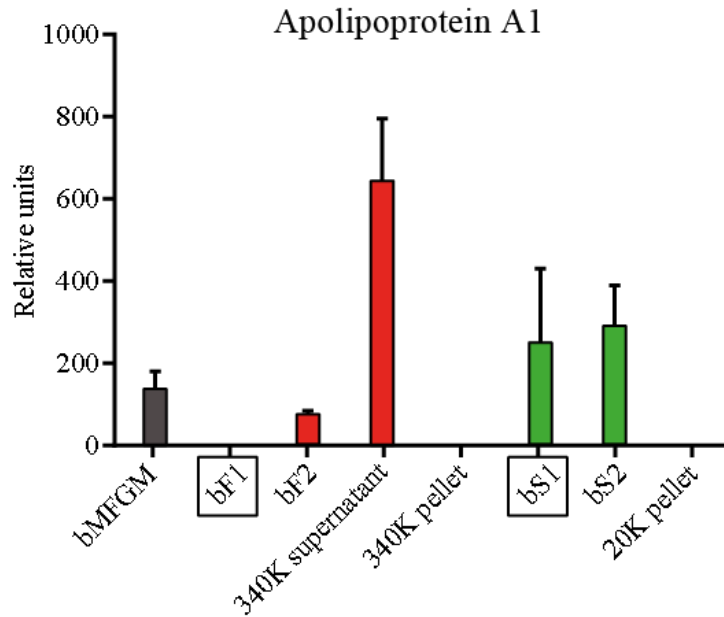


Fig. S3 Relative Apolipoprotein A1 levels by LC-MS in bovine milk fractions from the Fluff layer (red) and Milk Serum (green) MEV isolation

The peptide for quantification of apolipoprotein A1 (ApoA1) was selected solely on a theoretical level and the optimization of transitions was performed on non-labelled synthetic peptides (Thermo Fisher). Digestion, separation, detection and analysis of the resulting peptides were performed as described in the materials and methods section. MS transitions for the selected peptide can be found in **Table S2**.

LC-MS: Liquid Chromatography-Mass Spectrometry, bF1: Bovine Fluff MEV, bF2: Bovine Fluff 2. SEC peak, bS1: Bovine Milk Serum MEV, bS2: Bovine Milk Serum 2. SEC peak.

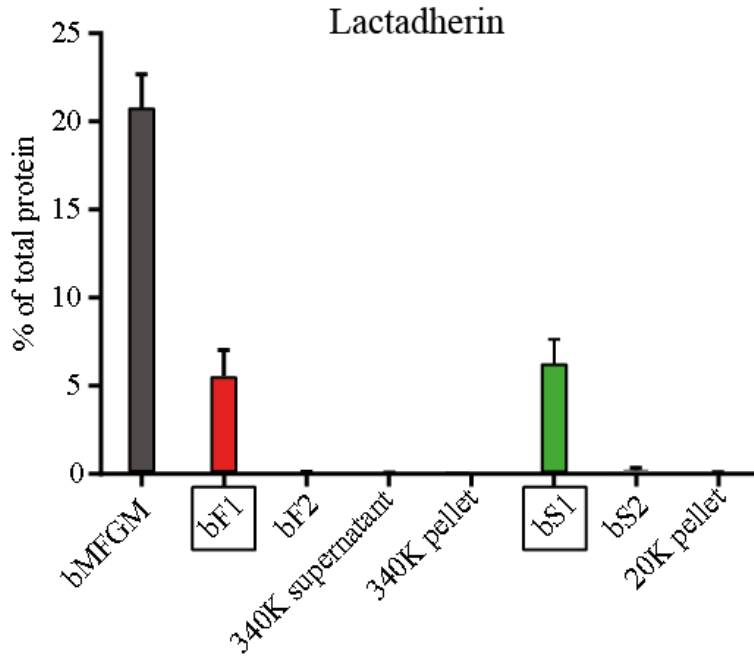


Fig. S4 Absolute lactadherin quantification by LC-MS in bovine milk fractions from the Fluff layer (red) and Milk Serum (green) MEV isolation

Bovine lactadherin (ID:Q95114) was quantified absolutely in milk fractions using the isotope labelled lactadherin peptide (VTGIITQGA[R(¹³C₆ ¹⁵N₄)] as internal standard. Absolute quantification was based on a standard curve of non-labelled synthetic lactadherin peptide added the labelled peptide and quantified by selective reaction monitoring by LC-MS. Digestion, separation, detection and analysis of the resulting peptide was performed as described in the materials and methods section. MS transitions for the selected peptide can be found in **Table S2**.

LC-MS: Liquid Chromatography-Mass Spectrometry, bF1: Bovine Fluff MEV, bF2: Bovine Fluff 2. SEC peak, bS1: Bovine Milk Serum MEV, bS2: Bovine Milk Serum 2. SEC peak.

Abbreviation	Average RCF	Speed (RPM)	Rotor type	Adjusted <i>k</i> factor	Run time (minutes)
-	3400	4000 ^B	H-6000A ¹ Swinging bucket, Sorvall	12240.6	35
-	1250	4000 ^H	Fiberlite F21-8x50y ² Fixed angle, Thermo Scientific	18474.1	35
20K	20000	16000	Fiberlite F21-8x50y ² Fixed angle, Thermo Scientific	1154.6	60
340K	340000	68000	Type 70 Ti fixed angle ³ Beckman Coulter	46.3	60

Table S1. Relative centrifugal forces, speed, rotor type, adjusted clearing factor and run times applied in the isolation approaches. Tubes were filled to nominal capacity. Applied centrifuges: 1. Sorvall RC5C, 2. Sorvall RC3C plus, 3. Beckman L8-70M ultracentrifuge. ^BBovine milk, ^HHuman milk.

Pellet-free isolation of human and bovine milk extracellular vesicles by size-exclusion chromatography

Protein	Protein ID	Peptide position	Peptide	Fragmentor [V]	Transition [m/z] [M+2H] ²⁺ →[M+H] ⁺			
					Quantifier ion	CE [mV]	Qualifier ion	CE [mV]
CD63 antigen	Q9XSK2	157-163	ILAVTNK	74	379.7 → 645.4	7	379.7 → 532.3	5
CD9 antigen	P30932	169-175	NLIDSLK	127	401.7 → 575.3	5	401.7 → 462.3	8
Lactadherin	Q95114	339-348	VTGIITQGAR	124	508.3 → 645.4	13	508.3 → 532.3	14
Lactadherin (labelled peptide)	Q95114	-	VTGIITQGA[R(13C6 15N4)]	124	513.3 → 655.4	13	513.3 → 542.3	14
β-casein	P02666	185-191	VLPVPQK	86	391.3 → 372.2	15	391.3 → 568.3	6
α-S1 casein	P02662	106-115	YLGYLEQLLR	143	634.9 → 529.3	27	634.9 → 401.3	22
α-S2 casein	P02663	130-140	NAVPIPTLNR	118	598.8 → 600.3	23	598.8 → 701.4	21
Apolipoprotein A-I	P15497	229-237	AKPVLEDLR	136	520.8 → 841.5	13	520.8 → 645.4	17

Table S2. Mass spectrometry transitions