**ELECTRONIC SUPPLEMENTARY MATERIALS**

**Human colostrum in vitro protein digestion: Peptidomics by LC-Orbitrap-HRMS and prospecting for bioactive peptides via bioinformatics**

Isabele B. Campanhon1,2, Paula Fernandes de Aguiar3, Flávia Fioruci Bezerra4, Márcia R. Soares1,\*, Alexandre Guedes Torres2

Authors’ affiliations:

1 Department of Biochemistry, Institute of Chemistry, Federal University of Rio de Janeiro, Brazil

2 Lipid Biochemistry and Lipidomics Laboratory and Laboratory of Food Science and Nutritional Biochemistry, Institute of Chemistry, Federal University of Rio de Janeiro, Brazil

3 Laboratory of Chemometrics (LABQUIM), Department of Analytical Chemistry, Institute of Chemistry, Federal University of Rio de Janeiro, Brazil

4 Department of Basic and Experimental Nutrition, Nutrition Institute, State University of Rio de Janeiro, Brazil

**Supplementary Table 1**. Formulation of the artificial digestive fluids*\** used for the *in vitro* digestion of colostrum.

**Supplementary Table 2.** Characteristics (mean ± SD) of Brazilian lactating women and their new-borns (girls, *n*= 13 and boys, *n* = 11) participating in the study, during colostrum collection.

**Supplementary Table 3.** Precursor proteins of the peptides that are released during each phase of *in vitro* colostrum digestion.

**Supplementary Table 4.** Details of the proteolytic activity during *in vitro* digestion of colostrum predicted using *Enzyme Predictor*.

**Supplementary Table 5.** Prediction by the ClassAMP tool of antimicrobial activity of peptides released during *in vitro* digestion of human colostrum. (binded Excel spreadsheet)

**Supplementary Table 1**. Formulation of the artificial digestive fluids*\** used for the *in vitro* digestion of colostrum.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Components** | **Saliva** | **Gastric Fluid** | **Duodenal Fluid** | **Bile** |
| **Inorganic** | KCl, 89.6 g/LNaCl, 175.3 g/LNaHCO3, 84.7 g/LNaH2PO4, 88.8 g/LNa2SO4, 57.0 g/LKSCN, 20.0 g/L | KCl, 89.6 g/LNaCl, 175.3 g/LCaCl2, 16.65 g/LNaH2PO4, 88.8 g/LNH4Cl, 30.6 g/L3.25 mL HCl, 37% g/g | KCl, 89.6 g/LNaCl, 175.3 g/LMgCl2, 5.0 g/LNaHCO3, 84.7g/LKH2PO4, 8.0 g/L90µL HCl, 37% g/g | KCl, 89.6 g/LNaCl, 175.3 g/LNaHCO3, 84.7 g/L75µL HCl, 37% g/g |
| **Organic** | Urea, 25.0 g/L | Urea, 25.0 g/LGlucose, 65.0 g/LGlucuronic acid, 2.0 g/LGlucosamine, hydrochloride, 33.0 g/L | Urea, 25.0 g/L | Urea, 25.0 g/L |
| **Added to the digestive mixture** | α-amylase, 290 mg mucin, 25 mguric acid, 15 mg  | BSA, 1.0 gmucin, 3.0 gpepsin, 2.5 g | BSA, 1.0 gCaCl2, 16.65 g/Llipase, 1.5 gpancreatin, 9.0 g  | BSA, 1.8 gCaCl2, 16.65 g/Lswine bile, 30g |
| **pH** | 6.8 ± 0.2 | 4.5 ± 0.2 | 7.5 ± 0.2 | 7.5 ± 0.2 |

\* The solutions of inorganic and organic components were prepared separately in bi-distilled water. After adjusting pH of each artificial digestive fluid with 1.0 mol/L HCl or 1.0 mol/L NaOH, solutions were made to 50 mL in a volumetric flask with bi-distilled water. The inorganic and organic solutions were mixed, enzymes and other components were added and dissolved by heating at 37 ºC under stirring. Whenever necessary, pH of each artificial digestive fluid was adjusted to the appropriate value in each digestion phase.

**Supplementary Table 2.** Characteristics (mean ± SD) of Brazilian lactating women and their new-borns (girls, *n*= 13 and boys, *n* = 11) participating in the study, during colostrum collection.

|  |  |
| --- | --- |
| **Characteristics (*n*= 24)** | **Mean ± SD** |
| Maternal age (years) | 26.0 ± 5.3 |
| Lactational period (days) | 2.0 ± 1.3 |
| Gestational age at birth (weeks) | 39.5 ± 1.7 |
| New-born weight at birth (kg) | 3.16 ± 0.60 |

**Supplementary Table 3.** Precursor proteins of the peptides that are released during each phase of *in vitro* colostrum digestion.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Accession** | **Description** | **Unique Peptides** | **MW [kDa]** | **p*I*** | **Oral Phase** | **Gastric Phase 35’** | **Gastric Phase 65’** | **Duodenal Phase** |
| P05814 | Beta-casein | 921 | 25.366 | 5.78 |  |  |  |  |
| P07498 | Kappa-casein | 189 | 20.293 | 8.92 |  |  |  |  |
| P47710 | Alpha-S1-casein | 5 | 21.658 | 5.38 |  |  |  |  |
| P47710-2 | Isoform 2 of Alpha-S1-casein | 1 | 21.53 | 5.38 |  |  |  |  |
| P01833 | Polymeric immunoglobulin receptor | 155 | 83.232 | 5.74 |  |  |  |  |
| P10909-2 | Isoform 2 of Clusterin | 110 | 57.796 | 6.68 |  |  |  |  |
| P24821-4 | Isoform 4 of Tenascin | 126 | 230.72 | 4.91 |  |  |  |  |
| P01876 | Immunoglobulin heavy constant alpha 1 | 23 | 37.631 | 6.51 |  |  |  |  |
| P0DOX2 | Immunoglobulin alpha-2 heavy chain | 16 | 48.904 | 6.81 |  |  |  |  |
| P10451-5 | Isoform 5 of Osteopontin | 46 | 33.823 | 4.61 |  |  |  |  |
| P01024 | Complement C3 | 48 | 187.03 | 6.4 |  |  |  |  |
| P19835 | Bile salt-activated lipase | 38 | 79.272 | 5.34 |  |  |  |  |
| Q08431 | Lactadherin | 32 | 43.095 | 8.15 |  |  |  |  |
| P0DOX7 | Immunoglobulin kappa light chain | 32 | 23.364 | 7.17 |  |  |  |  |
| P0DOY2 | Immunoglobulin lambda constant 2 | 16 | 11.287 | 7.24 |  |  |  |  |
| P02768 | Serum albumin | 15 | 69.321 | 6.28 |  |  |  |  |
| P0DOX6 | Immunoglobulin mu heavy chain | 2 | 63.446 | 7.87 |  |  |  |  |
| P01871-2 | Isoform 2 of Immunoglobulin heavy constant mu | 1 | 51.891 | 6.15 |  |  |  |  |
| P0C0L4 | Complement C4-A | 21 | 192.664 | 7.08 |  |  |  |  |
| P25311 | Zinc-alpha-2-glycoprotein | 16 | 34.237 | 6.05 |  |  |  |  |
| P60709 | Actin, cytoplasmic 1 | 3 | 41.71 | 5.48 |  |  |  |  |
| P68032 | Actin, alpha cardiac muscle 1 | 2 | 41.992 | 5.39 |  |  |  |  |
| P15291 | Beta-1,4-galactosyltransferase 1 | 9 | 43.892 | 8.65 |  |  |  |  |
| P23280-2 | Isoform 2 of Carbonic anhydrase 6 | 7 | 35.343 | 8.4 |  |  |  |  |
| Q8NFU4 | Follicular dendritic cell secreted peptide | 8 | 9.694 | 9.32 |  |  |  |  |
| Q13410 | Butyrophilin subfamily 1 member A1 | 8 | 58.923 | 5.54 |  |  |  |  |
| P01009 | Alpha-1-antitrypsin | 7 | 46.707 | 5.59 |  |  |  |  |
| P47989 | Xanthine dehydrogenase/oxidase | 7 | 146.33 | 7.66 |  |  |  |  |
| P01011 | Alpha-1-antichymotrypsin | 5 | 47.621 | 5.52 |  |  |  |  |
| A0A0B4J1Y9 | Immunoglobulin heavy variable 3-72 | 4 | 13.194 | 7.85 |  |  |  |  |
| A0A0B4J1V0 | Immunoglobulin heavy variable 3-15 | 4 | 12.917 | 8.62 |  |  |  |  |
| P61769 | Beta-2-microglobulin | 4 | 13.706 | 6.52 |  |  |  |  |
| P0DOX8 | Immunoglobulin lambda-1 light chain | 1 | 22.816 | 6.76 |  |  |  |  |
| **Accession** | **Description** | **Unique Peptides** | **MW [kDa]** | **p*I*** | **Oral Phase** | **Gastric Phase 35’** | **Gastric Phase 65’** | **Duodenal Phase** |
| Q9BTM1-2 | Isoform 2 of Histone H2A.J | 5 | 16.1 | 10.23 |  |  |  |  |
| P16401 | Histone H1.5 | 4 | 22.566 | 10.92 |  |  |  |  |
| Q5QNW6-2 | Isoform 2 of Histone H2B type 2-F | 6 | 14.832 | 10.35 |  |  |  |  |
| P01034 | Cystatin-C | 4 | 15.789 | 8.75 |  |  |  |  |
| P00709 | Alpha-lactalbumin | 6 | 16.214 | 5.0 |  |  |  |  |
| A0A075B6K4 | Immunoglobulin lambda variable 3-10 | 4 | 12.433 | 4.83 |  |  |  |  |
| P05109 | Protein S100-A8 | 4 | 10.828 | 7.03 |  |  |  |  |
| P02750 | Leucine-rich alpha-2-glycoprotein | 5 | 38.154 | 6.95 |  |  |  |  |
| P68104 | Elongation factor 1-alpha 1 | 5 | 50.109 | 9.01 |  |  |  |  |
| P08571 | Monocyte differentiation antigen CD14 | 4 | 40.051 | 6.23 |  |  |  |  |
| O00560 | Syntenin-1 | 4 | 32.424 | 7.53 |  |  |  |  |
| Q08380 | Galectin-3-binding protein | 3 | 65.289 | 5.27 |  |  |  |  |
| P05164-3 | Isoform H7 of Myeloperoxidase | 3 | 87.193 | 9.07 |  |  |  |  |
| P10412 | Histone H1.4 | 1 | 21.852 | 11.03 |  |  |  |  |
| P16233 | Pancreatic triacylglycerol lipase | 2 | 51.124 | 6.73 |  |  |  |  |
| Q6WN34-2 | Isoform 2 of Chordin-like protein 2 | 3 | 49.643 | 7.14 |  |  |  |  |
| P16403 | Histone H1.2 | 1 | 21.352 | 10.93 |  |  |  |  |
| P12273 | Prolactin-inducible protein | 3 | 16.562 | 8.05 |  |  |  |  |
| P62328 | Thymosin beta-4 | 2 | 5.05 | 5.06 |  |  |  |  |
| P01703 | Immunoglobulin lambda variable 1-40 | 3 | 12.294 | 5.74 |  |  |  |  |
| P06702 | Protein S100-A9 | 2 | 13.234 | 6.13 |  |  |  |  |
| P07737 | Profilin-1 | 2 | 15.045 | 8.27 |  |  |  |  |
| P80748 | Immunoglobulin lambda variable 3-21 | 2 | 12.438 | 5.29 |  |  |  |  |
| P02765 | Alpha-2-HS-glycoprotein | 1 | 39.3 | 5.72 |  |  |  |  |
| P04746 | Pancreatic alpha-amylase | 2 | 57.67 | 7.05 |  |  |  |  |
| P14174 | Macrophage migration inhibitory factor | 2 | 12.468 | 7.88 |  |  |  |  |
| Q8NBJ4 | Golgi membrane protein 1 | 2 | 45.306 | 4.97 |  |  |  |  |
| P04406 | Glyceraldehyde-3-phosphate dehydrogenase | 2 | 36.03 | 8.46 |  |  |  |  |
| Q99541 | Perilipin-2 | 2 | 48.045 | 6.8 |  |  |  |  |
| P62805 | Histone H4 | 1 | 11.36 | 11.36 |  |  |  |  |
| P08246 | Neutrophil elastase | 2 | 28.5 | 9.35 |  |  |  |  |
| Q6UX06 | Olfactomedin-4 | 2 | 57.244 | 5.69 |  |  |  |  |
| P36268-3 | Isoform 3 of Inactive gamma-glutamyltranspeptidase 2 | 2 | 62.082 | 7.33 |  |  |  |  |
| A0A0B4J1X8 | Immunoglobulin heavy variable 3-43 | 1 | 13.068 | 5.41 |  |  |  |  |
| A0A075B6H9 | Immunoglobulin lambda variable 4-69 | 1 | 12.765 | 6.51 |  |  |  |  |
| O14613 | Cdc42 effector protein 2 | 1 | 22.469 | 5.12 |  |  |  |  |
| A0A0C4DH38 | Immunoglobulin heavy variable 5-51 | 1 | 12.666 | 8.27 |  |  |  |  |
| P05387 | 60S acidic ribosomal protein P2 | 1 | 11.658 | 4.54 |  |  |  |  |
| **Accession** | **Description** | **Unique Peptides** | **MW [kDa]** | **p*I*** | **Oral Phase** | **Gastric Phase 35’** | **Gastric Phase 65’** | **Duodenal Phase** |
| Q9H173 | Nucleotide exchange factor SIL1 | 1 | 52.052 | 5.36 |  |  |  |  |
| Q5TBA9 | Protein furry homolog | 1 | 338.659 | 5.99 |  |  |  |  |
| P02649 | Apolipoprotein E | 1 | 36.132 | 5.73 |  |  |  |  |
| P0DOX3 | Immunoglobulin delta heavy chain | 1 | 56.189 | 8.02 |  |  |  |  |
| P0DJI8 | Serum amyloid A-1 protein | 1 | 13.524 | 6.79 |  |  |  |  |
| A0A0A0MS15 | Immunoglobulin heavy variable 3-49 | 1 | 13.048 | 8.62 |  |  |  |  |
| P01619 | Immunoglobulin kappa variable 3-20 | 1 | 12.549 | 4.96 |  |  |  |  |
| P49327 | Fatty acid synthase | 1 | 273.254 | 6.44 |  |  |  |  |
| P08754 | Guanine nucleotide-binding protein G(k) subunit alpha | 1 | 40.506 | 5.69 |  |  |  |  |
| A0A0C4DH42 | Immunoglobulin heavy variable 3-66 | 1 | 12.69 | 8.16 |  |  |  |  |
| P31146 | Coronin-1A | 1 | 50.994 | 6.68 |  |  |  |  |
| Q9NZH0 | G-protein coupled receptor family C group 5 member B | 1 | 44.766 | 8.24 |  |  |  |  |
| P01591 | Immunoglobulin J chain | 1 | 18.087 | 5.24 |  |  |  |  |
| Q02878 | 60S ribosomal protein L6 | 1 | 32.708 | 10.58 |  |  |  |  |
| P07996 | Thrombospondin-1 | 1 | 129.3 | 4.94 |  |  |  |  |
| P0DP06 | Immunoglobulin heavy variable 4-30-4 | 1 | 13.148 | 9.26 |  |  |  |  |
| P0DOX5 | Immunoglobulin gamma-1 heavy chain | 1 | 49.299 | 8.72 |  |  |  |  |
| P11021 | 78 kDa glucose-regulated protein | 1 | 72.288 | 5.16 |  |  |  |  |
| Q9Y240 | C-type lectin domain family 11 member A | 1 | 35.673 | 5.16 |  |  |  |  |
| A0A0B4J1V6 | Immunoglobulin heavy variable 3-73 | 1 | 12.849 | 9.17 |  |  |  |  |
| P04004 | Vitronectin | 1 | 54.271 | 5.8 |  |  |  |  |
| A0A0A0MRZ8 | Immunoglobulin kappa variable 3D-11 | 1 | 12.617 | 5.29 |  |  |  |  |
| P06858 | Lipoprotein lipase | 1 | 53.129 | 8.15 |  |  |  |  |
| Q86Y38 | Xylosyltransferase 1 | 1 | 107.501 | 9.22 |  |  |  |  |
| Q15596 | Nuclear receptor coactivator 2 | 1 | 159.056 | 6.64 |  |  |  |  |
| P78364 | Polyhomeotic-like protein 1 | 1 | 105.468 | 9.03 |  |  |  |  |
| Q96PN7 | Transcriptional-regulating factor 1 | 1 | 132.173 | 6.74 |  |  |  |  |
| Q9NPB3 | Calcium-binding protein 2 | 1 | 24.466 | 4.7 |  |  |  |  |
| I3L1E1 | Uncharacterized protein C19orf84 | 1 | 19.602 | 7.43 |  |  |  |  |
| P15941-2 | Isoform 2 of Mucin-1 | 1 | 122.868 | 7.56 |  |  |  |  |
| Q6PCB0 | von Willebrand factor A domain-containing protein 1 | 1 | 46.775 | 7.68 |  |  |  |  |
| Q8N5K1 | CDGSH iron-sulfur domain-containing protein 2 | 1 | 15.268 | 9.61 |  |  |  |  |
| P05413 | Fatty acid-binding protein, heart | 1 | 14.849 | 6.8 |  |  |  |  |
| P06396 | Gelsolin | 1 | 85.644 | 6.28 |  |  |  |  |
| P02647 | Apolipoprotein A-I | 1 | 30.759 | 5.76 |  |  |  |  |

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Accession** | **Description** | **Unique Peptides** | **MW [kDa]** | **p*I*** | **Oral Phase** | **Gastric Phase 35’** | **Gastric Phase 65’** | **Duodenal Phase** |
| Q9UN76 | Sodium- and chloride-dependent neutral and basic amino acid transporter B(0+) | 1 | 72.105 | 8.19 |  |  |  |  |
| P26583 | Presente mobility group protein B2 | 1 | 24.019 | 7.81 |  |  |  |  |
| Q9UNQ2 | Probable dimethyladenosine transferase | 1 | 35.214 | 9.99 |  |  |  |  |
| B0YJ81 | Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 1 | 1 | 32.367 | 9.04 |  |  |  |  |
| Q5VTT5 | Myomesin-3 | 1 | 162.088 | 6.23 |  |  |  |  |
| Q5TGU0 | Translocator protein 2 | 1 | 19.116 | 8.31 |  |  |  |  |

**Supplementary Table 4.** Details of the proteolytic activity during *in vitro* digestion of colostrum predicted using *Enzyme Predictor*.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Enzyme | N-terminal cleavage count | C-terminal cleavage count | Total cleavage | Number of proteins cleaved |
| Plasmin | 371 | 521 | 892 | 188 |
| Trypsin | 369 | 521 | 890 | 188 |
| Chymotrypsin | 566 | 416 | 982 | 157 |
| Cathepsin D | 585 | 445 | 1030 | 130 |
| Pepsin (pH 1.3) 1 | 390 | 419 | 809 | 134 |
| Pepsin (pH 1.3) 2 | 460 | 297 | 757 | 153 |
| Elastase | 290 | 238 | 528 | 126 |
| Pepsin (pH >2) 1 | 289 | 355 | 644 | 121 |
| Pepsin (pH >2) 2 | 303 | 224 | 527 | 127 |
| Glutamyl Endopeptidase | 73 | 122 | 195 | 60 |