UNIVERSIDADE FEDERAL DE VIÇOSA

RAYANE MONIQUE BERNARDES LOCH

PROTEÔMICA DO LEITE HUMANO E INFLUÊNCIA DAS FASES DE LACTAÇÃO E DO SEXO DOS LACTENTES

VIÇOSA - MINAS GERAIS

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Dissertação apresentada à Universidade Federal de Viçosa como parte das exigências do Programa de Pós-Graduação em Bioquímica Aplicada para obtenção do título de *Magister Scientiae*.

Orientadora: Maria Cristina B. Pereira

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Rayane Monique Bernardes Loch
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“A ciência sempre será uma busca, jamais uma descoberta.

É uma viagem, nunca uma chegada”

Karl Popper

BIOGRAFIA

Rayane Monique Bernardes Loch, filha de Maria José Martins Bernardes e Roberto Amaral Bernardes, nasceu em 20 de julho de 1993, em Divinópolis/Minas Gerais.

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RESUMO

LOCH, Rayane Monique Bernardes, M.Sc., Universidade Federal de Viçosa, agosto de 2020. **Proteômica do leite humano e influência das fases de lactação e do sexo dos lactentes**. Orientadora: Maria Cristina Baracat Pereira.

O leite humano (HM) é um alimento completo que pode atender a todas as demandas nutricionais do recém-nascido. Em sua composição, há uma quantidade expressiva de proteínas bioativas que possuem atividade antimicrobiana e que também atuam no desenvolvimento digestivo e neurológico do recém-nascido. No entanto, no Brasil, as diretrizes sobre a distribuição de HM nos bancos de leite são baseadas principalmente no conteúdo energético. Existem alguns estudos em que macromoléculas do HM foram analisadas, mostrando diferenças na composição entre os grupos por alguns fatores, como a fase de lactação e o sexo do recém-nascido. No entanto, abordagens proteômicas que relacionam o impacto desses dois fatores na composição do HM ainda são escassas. Nossa hipótese é que esses fatores afetam a composição proteica do HM, levando à existência de proteínas específicas e diferencialmente abundantes, o que pode ser importante para um melhor desenvolvimento do bebê. Portanto, buscando responder a essas perguntas, este estudo compara, por meio de abordagens proteômicas (nano-LC/MS-MS), as diferenças apresentadas no HM nas fases colostro e maduro, e entre o sexo dos recém-nascidos. Como resultado, obtivemos 142 proteínas identificadas, 13 das quais ainda não haviam sido identificadas no HM. Além disso, obtivemos 54 proteínas diferencialmente abundantes entre as fases da lactação e 42 na comparação do sexo do recém-nascido. Essas proteínas estão principalmente relacionadas aos processos metabólicos e de defesa imunológica, sugerindo que existem necessidades nutricionais específicas para lactentes do sexo feminino e masculino, e entre as fases colostro e maduro. Com base em nossos resultados, destacamos a relevância de desenvolver estratégias específicas para a distribuição do HM nos bancos de leite, além de mudanças nas formulações infantis para aproximar-se da composição do HM.

Palavras-chave: Leite Humano. Proteômica comparativa. Sexo do recém-nascido. Fases da lactação.

ABSTRACT

LOCH, Rayane Monique Bernardes, M.Sc., Universidade Federal de Viçosa, August, 2020. **Proteomics of human milk and the influence of lactation phase and newborn's sex**. Advisor: Maria Cristina Baracat Pereira.

Human milk (HM) is a complete food that can meet all the nutritional and energetic demands of the newborn. In its composition there is an expressive amount of bioactive proteins that have antimicrobial activity and that also play important roles in digestive and neurological development. However, in Brazil, the guidelines on the distribution of HM in milk banks are based especially on energy content. There are some studies in which other HM macromolecules were analyzed, showing differences in composition between groups for some factors, such as the lactation phase and the newborn's sex. However, proteomic approaches relating the impact of both these factors on HM composition are still scarce. Our hypothesis is that these factors affect the protein composition of HM, leading to the existence of specific and differentially abundant proteins, which may be important for a better development of the infant. Therefore, seeking to answer these questions, this study compares, through proteomic approaches (nano-LC/MS-MS), the differences presented in HM in the colostrum and mature phases, and between the sex of newborns. As a result, we obtained 142 identified proteins, 13 of which had not yet been described in HM. In addition, we obtained 54 differentially abundant proteins between the lactation phases, and 42 by comparing the newborn´s sex. These proteins were mainly related to metabolic and immune defense processes, suggesting that there are specific nutritional needs for females and males infants, and between the colostrum and mature phases. Based on our results, we point out the need to develop specific strategies for the distribution of HM in milk banks, in addition to changes on infant formulations to bring it closer to the composition of HM.

Keywords: Human milk. Comparative proteomics. Newborn's sex. Lactation phases.

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LISTA DE ABREVIATURAS

CHAPS 3-[(3-Cholamidopropyl) dimethylammonio] -1-propanesulfonate hydrate

Co colostrum phase

DTT dithiothreitol

F female

FC female colostrum

FM female mature

HM human milk

Ig immunoglobulin secretory

LC-MS Liquid Chromatography Coupled to Mass Spectrometry

M males

Ma mature phase

MC male colostrum

MM male mature

PCA multivariate principal component analysis

SDS PAGE sodium dodecyl sulphate–polyacrylamide gel electrophoresis

SUMÁRIO

1. INTRODUÇÃO

O aleitamento materno se destaca na prevenção de mortes infantis, leva à redução dos riscos de mortalidade associados ao parto prematuro, e atua no desenvolvimento do sistema imune e cognitivo do recém-nascido, dentre outras vantagens para o lactente. Em relação à lactante, a amamentação auxilia na manutenção de sua saúde física e mental. O leite humano é resultado de um processo evolutivo ao longo de mais de 200 milhões de anos, apresentando em sua composição proteínas com alto valor nutricional que são importantes fontes de energia, crescimento e desenvolvimento de recém-nascidos (Brunser, 2018).

Recomenda-se o aleitamento materno por dois anos ou mais, sendo exclusivo nos primeiros seis meses de vida (WHO, 2013). Na impossibilidade da amamentação com leite humano, outros tipos de leite quando oferecidos aos lactentes podem levar ao desenvolvimento de doenças e alergias, e oferecer riscos a alguns órgãos, como danos no intestino, cérebro e rins (Andreas; Kampmann; Mehring, 2015 ; Jakaitis; Denning, 2014 ; Munblit et al., 2017).

As modificações do perfil proteico do leite humano em suas diferentes fases de lactação - colostro, transição e maduro - estão relacionadas especialmente com as necessidades do lactente em cada período de vida, buscando suprir as demandas fisiológicas (Calil; Falcão, 2003). Isso pode ser observado no leite humano, em que suas características inicialmente são mais voltadas para o desenvolvimento imunológico do recém-nascido, e posteriormente se altera para um perfil mais nutricional (Andreas; Kampmann; Mehring, 2015).

É expressiva a quantidade de proteínas bioativas no leite humano que apresentam atividade antimicrobiana e anti-inflamatória contra bactérias, vírus e fungos, e que atuam no desenvolvimento digestivo e neurológico. Assim, há grande interesse na busca de melhorias da qualidade das fórmulas infantis, não apenas para adaptar a concentração de macronutrientes e micronutrientes, mas também para a inserção de compostos bioativos para tornar as fórmulas o mais semelhante possível ao leite humano, oferecendo uma proteção constitutiva similar.

Segundo Garcia-Rodenas et al. (2018), são escassos os estudos relacionados com as proteínas presentes no leite humano em que haja uma caracterização proteica geral. Alhindi et al. (2019) e Dafaallah et al. (2018) também sugerem limitações de estudos em que o gênero do lactente é tido como parâmetro de avaliação do perfil proteico. Melo et al. (2020) relataram que foram encontrados apenas cinco estudos envolvendo o gênero do lactente, sendo que nenhum deles estava relacionado à proteômica.

Sendo assim, o presente trabalho se mostra relevante ao possibilitar que se possa comparar as diferenças apresentadas no leite em diferentes fases de lactação, e a variabilidade existente entre o leite produzido por mães de recém-nascidos do sexo feminino e masculino. Essa pesquisa contribui para uma melhor compreensão das funções das proteínas presentes no leite e além disso pode ser aplicada ao desenvolvimento de formulações infantis que melhor atendam às necessidades dos lactentes em casa fase do desenvolvimento, tornando o conteúdo proteico destas mais rico, e pode auxiliar a direcionar as recomendações da ingestão do leite disponível em bancos de leite humano.

Pretendeu-se nesse caracterizar o conteúdo proteico do leite humano nas diferentes fases de lactação, assim como avaliar as diferenças existentes entre o leite de mães de crianças do sexo feminino e masculino, de forma que se possa auxiliar o entendimento desses perfis proteicos e, posteriormente, no desenvolvimento de produtos biotecnológicos mais adequados a cada caso.

2. OBJETIVOS

2.1 Geral

Comparar o perfil proteico do leite humano em diferentes fases da lactação e sexo do lactente.

2.2 Específicos

a. Realizar análise proteômica para caracterização do perfil proteico geral do leite humano;

b. Realizar análise proteômica para caracterização das proteínas diferencialmente abundantes do leite humano nas fases de colostro e maduro e no leite de mães de recém-nascidos do sexo masculino e feminino;

c. Classificar funcionalmente as proteínas do leite humano em amostras de mães de acordo com a fase de lactação e sexo do lactente.

3. REVISÃO DE LITERATURA

3.1 Leite humano

A Organização Mundial da Saúde recomenda aleitamento materno exclusivo por seis meses e complementado até os dois anos de idade ou mais. Até esse período de seis meses sendo alimentados apenas com o leite materno, a criança recebe nutrientes que suprem todas as suas necessidades nutricionais necessárias para o seu desenvolvimento, tornando-se desnecessários outros alimentos e líquidos. No entanto, mesmo após o início da alimentação complementar, o leite materno permanece como uma importante fonte energética que atende a diversas demandas nutricionais da criança. Estima-se que o leite materno seja capaz de contribuir com cerca de metade dessas demandas até o primeiro ano de vida, e até um terço delas no segundo ano (WHO, 2013).

Isso ocorre porque o leite humano é o único alimento capaz de satisfazer todas as necessidades de um recém-nascido, fornecendo carboidratos, proteínas, lipídios e vitaminas, além de uma variedade de compostos bioativos, como fatores de crescimento, hormônios, citocinas e compostos antimicrobianos (Garwolinska et al., 2018). Os lipídios atuam principalmente no metabolismo de energia, composição de membranas, desenvolvimento cerebral; carboidratos, no metabolismo energético e na glicosilação de proteínas e lipídios; e as proteínas, atuam em mecanismos de defesa, nutricional, imunológicos e de crescimento (Roncada et al., 2013).

Em relação à composição do leite humano, ela é alterada e influenciada por diversos fatores, como genéticos, geográficos, ambientais, alimentação da mãe, e frequência e horário da retirada do leite (Bardanzellu; Fanos; Reali, 2017). Estima-se uma composição média de 3,2% (p/v) de proteínas, 4% de lipídeos, 5% de carboidrato e 0,7% de sais minerais. As estimativas de energia variam de 65 a 70 kcal / dL e estão correlacionadas com o teor de gordura do leite humano (Séverin; Wenshui, 2005; Fields; Demerath, 2013).

A amamentação também traz para a mãe diversos benefícios, como o maior vínculo com o recém-nascido, redução do risco de câncer de mama e auxílio na perda da gordura obtida na gestação (Prell; Koletzko, 2016). Apesar de todos os benefícios apresentados, é comum que o aleitamento materno cesse antes dos seis meses. Os principais fatores de desmame são idade materna (adolescência), nível

socioeconômico e escolaridade, experiência anterior, e falta de suporte e incentivo pelos profissionais da saúde e família (Leone; Sadeck, 2012).

Estudos como de Kull et al. (2004) e Smilowitz et al. (2013) também evidenciaram outros benefícios, indicando que o leite materno diminui a taxa de asma e diabetes em recém-nascidos. Apesar disso, no Brasil, a duração média do aleitamento exclusivo é de 54 dias, e apenas 41% das crianças menores de seis meses são alimentadas exclusivamente com leite materno (Ministério da Saúde, 2018).

3.2 Aspectos biológicos das proteínas do leite humano

O conteúdo proteico do leite é uma rica fonte de aminoácidos e nitrogênio que são necessários para o crescimento e desenvolvimento de um recém-nascido, e suas proteínas apresentam atividades na digestão de nutrientes, proteção contra patógenos e na resposta imune. As proteínas mais abundantes no leite humano são caseína, α-lactalbumina, lactotransferrina, imunoglobulina secretora (IgA), lisozima e albumina sérica (Lonnerdal, 2003).

Embora se tenha opções de fórmulas infantis para substituição do leite materno, essas não são capazes de fornecer a variedade de compostos e moléculas bioativas presentes no leite humano (Beck et al., 2015). Além disso o consumo de outros tipos de leite aumenta o risco do desenvolvimento de doenças e alergias no recém-nascido e pode ocasionar lesões no intestino que ainda não está totalmente desenvolvido (Passanha; Cervato-Mancuso; Silva, 2010).

O desenvolvimento e a colonização do intestino do recém-nascido têm influência do tipo de parto, visto que em partos normais ocorre o contato do bebê com as bactérias presentes na vagina e no cólon da mãe. Esse processo de colonização é o que estabelecerá uma microbiota saudável, e continua acontecendo por meio da amamentação (Jakaitis; Denning, 2014). Há também diferenças na microbiota entre crianças alimentada com leite humano e fórmulas infantis, em que há predomínio de bifidobactérias e lactobacilos para o primeiro grupo, e coliformes e bacteroides no segundo (Rolfe, 2000).

3.3 Dos estágios de lactação

As modificações na composição dos nutrientes presentes no leite humano nas fases de lactação – colostro, transição e maduro - estão relacionadas com as demandas fisiológicas que o organismo do lactente apresenta em cada etapa do seu desenvolvimento (Calil; Falcão, 2003).

A fase do leite colostro corresponde ao período do pós-parto até o sétimo dia após o nascimento, contém fatores imunológicos, nutricionais, e hormônios importantes para o desenvolvimento do recém-nascido (Palmer et al. 2006). Apresenta-se com maior viscosidade devido às concentrações mais elevadas de proteínas, minerais e vitaminas lipossolúveis, gera sua maior viscosidade, e com uma coloração mais amarelada decorrente da elevada quantidade de betacaroteno e resíduos de materiais celulares presentes nas glândulas e dutos mamários (Calil; Falcão, 2003).

As proteínas da fase colostro estão mais relacionadas ao sistema de defesa do lactente e há um decréscimo da concentração proteica com o decorrer do tempo em função do desenvolvimento do sistema imunológico da criança (Gidrewicz; Fenton, 2014). A fase colostro apresenta concentração relativamente baixa de lactose, indicando seu perfil mais imunológico que nutricional (Fields; Demerath, 2013).

O leite de transição corresponde àquele do período entre o sétimo e o décimo quinto dia após o nascimento, e seu perfil é semelhante ao da fase anterior (colostro). No entanto percebe-se um aumento em sua produção para acompanhar as necessidades nutricionais e de desenvolvimento do recém-nascido (Ballard; Morrow, 2013). Quanto aos seus componentes, há uma redução na concentração das proteínas, especialmente das imunoglobulinas, e aumento da lactose e dos lipídios, até atingir a fase de leite maduro (Matuhara; Nagamura, 2006).

A última fase chamada leite maduro e corresponde ao período do décimo quinto dia de nascimento em diante. Entretanto, de quatro a seis semanas após o parto, mudanças na composição do leite ainda ocorrem, e no fim desse período o leite é caracterizado como totalmente maduro (Ballard; Morrow, 2013). Nessa fase há maior produção de galactose e gordura, consequentemente aumentando sua

concentração, e em contrapartida há menor conteúdo de imunoglobulinas (Andreas; Kampmann; Mehring, 2015).

Essas alterações na composição das três fases refletem as mudanças nas necessidades do recém-nascido, que com o passar do tempo tem a taxa de crescimento reduzida e a capacidade de digerir e absorver os nutrientes aumentada (Donangelo; Trugo, 2003).

3.4 Proteínas do leite humano

A maioria das proteínas do leite são sintetizadas pelas glândulas mamárias e podem ser classificadas em três grupos: as caseínas, as proteínas do soro e as mucinas (Lonnerdal, 2013).

As caseínas são proteínas altamente glicosiladas, representam cerca de 40% das proteínas no leite humano e desempenham principalmente atividade imunológica no recém-nascido (Jakaitis; Denning, 2014). São encontradas como micelas, presentes na forma de dispersão coloidal. As micelas contribuem para a cor branca do leite e variam em tamanho desde 11 a 55 nm (Kunz et al., 1999). São altamente resistentes ao calor, sofrendo precipitação em pH igual ou inferior a cinco ou por ação enzimática. Existem três tipos de caseína no leite humano: α, β e κ-caseína. (Calil; Falcão, 2003).

A β - caseína é a caseína predominante encontrada no leite humano (50%). Quando seus aminoácidos constituintes são digeridos, formam-se fosfopeptídeos de caseína menores que facilitam a absorção de cálcio. Dessa forma, esses fosfopeptídeos de caseína contribuem para a elevada biodisponibilidade do cálcio a partir de leite humano (Lonnerdal, 2013). A presença de grande concentração de serinas fosforiladas também ajuda na absorção de cálcio. A molécula apresenta um alto conteúdo de prolina, o que lhe confere uma estrutura helicoidal solta e facilmente desnaturada (Goldfarb; Savadove; Inman, 1989).

A κ-caseína (cerca de 20 a 27% do grupo de caseína), é uma glicoproteína fortemente glicosilada com um peso molecular relatado variando de 30 a 40 kDa. Esta discrepância na massa molecular está associada às variações na glicosilação e com as dificuldades no isolamento da κ-caseína na forma pura (Stromqvist et al., 1985). Essa proteína pode promover o crescimento de bactérias benéficas e atuar

na inibição de bactérias ou vírus patogênicos, impedindo sua adesão às células epiteliais, e assim, prevenindo infecções (Kunz et al., 1999).

As proteínas do soro constituem, no leite humano, cerca de 60 a 90% de seu teor proteico total. As proteínas do soro são solúveis e seu teor no leite é muito alto, contendo principalmente imunoglobulinas, lactotransferrina e α-lactalbumina. A concentração dessas proteínas diminui à medida que a lactação progride (Kunz et al., 1999). A alfa-lactalbumina corresponde à principal proteína do leite, constituindo cerca de 40% das proteínas do soro do leite humano, atuando no transporte de ferro e na síntese de lactose na glândula mamária (Calil; Falcão, 2003).

Kunz; Bo Lonnerdal (1992) avaliaram a mudança de concentração das caseínas e proteínas de soro ao longo da lactação, e observaram que, após o parto, há uma menor concentração de caseínas, que terão aumento ao longo do tempo e da amamentação. As proteínas do soro, de forma diferente, mantêm sua concentração elevada ao longo do tempo. Logo, o conteúdo de aminoácidos presentes no leite humano varia de acordo com as fases de lactação.

As mucinas envolvem os glóbulos lipídicos no leite e contribuem apenas com uma pequena porcentagem do conteúdo total de proteínas do leite humano (1 a 2%) Como o teor de gordura do leite humano não varia durante o decorrer da lactação, sua concentração permanece quase constante em todas as fases de lactação, ao contrário das proteínas do soro e caseínas que tem sua concentração variada. As formulações infantis não apresentam proteínas pertencentes a esse grupo. (Lonnerdal, 2003).

3.5 Proteínas bioativas no leite humano

O leite humano possui um conjunto de proteínas bioativas, que apresentam outras funções além da nutrição, como defesa, atividades enzimáticas diversas, auxílio e aumento da absorção de nutrientes e estímulo de crescimento. Desde o nascimento, o recém-nascido já possui linhagens de bactérias em seu intestino, como a *Escherichia coli*, fazendo parte da sua flora normal. No entanto, o bebê ainda não possui o intestino totalmente maduro e, pela baixa acidez gástrica e menor atividade de enzimas digestivas, algumas dessas linhagens podem causar doença intestinal grave (Passanha; Cervato-Mancuso; Silva, 2010).

Grande quantidade de proteínas do leite humano possui atividades contra bactérias, vírus e fungos, podendo atuar de forma isolada ou em conjunto para conter os patógenos. Evidências apontam que atividades apresentadas por diferentes proteínas do leite humano em um mesmo patógeno possibilitam uma defesa ampla, que explica a menor incidência de infecção em crianças alimentadas por leite humano em relação às alimentadas com fórmula (Lonnerdal, 2003).

A seguir são apresentadas algumas proteínas do leite humano com atuação biológica para o recém nascido.

3.5.1. Lactotransferrina

A lactotransferrina é uma glicoproteína catiônica, com cerca de 80 kDa, presente no leite humano, e que apresenta propriedades bioativas. Há uma diminuição de sua concentração no leite humano com a evolução do período de lactação, estando, portanto, em maior concentração no colostro. Estudos apontam sua capacidade de ligação ao ferro, inibindo patógenos que o utilizariam para seu crescimento e sobrevivência (Haschke; Haiden; Thakkar, 2017). Em locais de infecção, temos o aumento da lactotransferrina, que apresentou atividade contra diversas bactérias, vírus e fungos (Berlutti et al., 2011). Sua expressão e secreção ocorre por meio de neutrófilos e células epiteliais e apresenta estrutura altamente conservada entre humanos, bovinos, ratos e porcos.

3.5.2 Lactoperoxidase

A lactoperoxidase é uma enzima oxidoredutase secretada pelas glândulas mamárias, salivares e outras mucosas, com cerca de 78 kDa, que age também na defesa antimicrobiana. Na presença de peróxido de hidrogênio, essa enzima catalisa a oxidação do tiocianato (componente natural do leite), levando à produção de um composto intermediário, o hipotiocianato**,** com propriedades antimicrobianas, especialmente contra bactérias Gram-positivas e Gram-negativas (Shin, Tomita e Lonnerdal, 2000; Séverin, Wenshui, 2005).

3.5.3 Lisozima

A lisozima é uma das principais enzimas que compõe o soro de leite humano, apresentando-se em elevada concentração (cerca de 15 vezes mais do que no leite

bovino). Possui atividade antibacteriana e pode atuar de forma individual contra bactérias por meio da clivagem de ligações glicosídicas β (1-4) da parede celular, ou de forma conjunta com a lactotransferrina. Nesse caso, a lactotransferrina gera lacunas na membrana da bactéria, permitindo que a lisozima entre mais facilmente e degrade o patógeno (Lonnerdal, 2013).

3.5.4 Imunoglobulinas (Ig)

A principal imunoglobulina (Ig) no leite humano é a IgA, embora também estejam presentes as IgG, IgM e IgD. A IgA do leite humano auxilia a proteger a criança contra uma variedade de patógenos por meio de bloqueio à adesão dos mesmos à mucosa (Kunz et al., 1999). A IgA exibe também uma boa resistência a alterações de pH e à digestão no estômago, decorrente de sua estrutura molecular (Calil; Falcão, 2003).

3.5.5 Haptocorrina

Haptocorrina é uma proteína que apresenta também propriedades antimicrobianas. Está associada à vitamina B-12 e a absorção da vitamina B-12. Em função da sua alta afinidade por vitamina B-12, é capaz de inibir o crescimento de agentes patogênicos por retenção dessa vitamina, não a deixando disponível para utilização pelos microrganismos (Lonnerdal, 2013).

3.5.6 Alfa-lactoalbumina

Alfa-lactoalbumina é uma subunidade da lactose, que não apresenta atividade catalítica de forma isolada. Apresenta um teor relativamente alto de triptofano, bem como lisina e cisteína, e é uma indutora de apoptose celular, agindo no sistema imunológico (Zhang et al., 2016)

3.5.7 Osteopontina

A osteopontina é uma proteína multifuncional, altamente glicosilada e fosforilada, com possíveis papéis na ativação imune, na inibição da calcificação ectópica, na adesão e na migração celular, na angiogênese e na remodelação óssea (Haschke; Haiden; Thakkar, 2017). Estudos apontam que essa proteína apresenta um papel de autoimunidade em diversos tipos de câncer e doenças

cardiovasculares, além de seu envolvimento na regulação do desenvolvimento do sistema cerebral (Demmelmair et al., 2017).

3.5.8 Citocinas

As citocinas correspondem a um grupo diversificado de proteínas presentes no leite humano e que auxiliam no controle de inflamações, diminuindo o efeito de infecções. As mais conhecidas são a interleucinas (IL-4, IL-5 IL-10 e IL-13), o fator de crescimento de transformação beta, interferon gama e o fator de necrose tumoral alfa. As interleucinas estão envolvidas na produção de imunoglobulina E, enquanto que o fator de crescimento de transformação beta na produção de imunoglobulina A. Dentre as classes de proteínas bioativas presentes no leite humano, as citocinas não são ainda tão bem estudadas, necessitando de mais informações (Lönnerdal, 2003; Passanha; Cervato-Mancuso; Silva, 2010).

3.5.9 Amilase

Amilase é uma enzima ativa em pH baixo e é relativamente estável contra a degradação da pepsina no estômago. Além de seu papel no auxílio da digestão, a amilase também pode apresentar atividade antibacteriana, atacando os polissacarídeos da parede celular de patógenos (Haschke; Haiden; Thakkar, 2017).

Outras importantes proteínas bioativas são as lipases e as caseínas, com função na absorção de nutrientes e estímulo de crescimento:

3.5.10 Lipase estimulada por sais biliares

Lipases estimuladas pelos sais biliares (BSSL) têm sido associadas à digestão dos lipídios do leite humano, particularmente em recém-nascidos prematuros e compreendem cerca de 1-2% do total de proteínas do leite. No lúmen intestinal, realizam a hidrólise da gordura do leite, vitamina A, colesterol e fosfolipídios, atuando na digestão e no metabolismo de lipídeos (Lonnerdal, 2013).

3.5.11 Caseína

As caseínas compreendem um grupo de subunidades proteicas que têm a propriedade de formar micelas estáveis com cálcio e fósforo, auxiliando no transporte de minerais, de grande importância para o metabolismo geral dos

lactentes. Existem vários subtipos de caseínas no leite humano, sendo as principais as frações beta-caseína (50%) e kappa-caseína (20 a 27%) (Calil; Falcão, 2003).

Neste contexto, este trabalho buscou contribuir para a determinação do perfil proteico do leite humano, e da abundância de proteínas presentes em cada variação. Essas informações são fundamentais para avaliação das estratégias utilizadas nos bancos de leite para atendimento de forma mais específica das necessidades individuais das crianças. Além disso, os resultados deste estudo podem ser aplicados para propostas de adequação de formulações infantis que possam melhor atender aos lactentes, diminuindo suas diferenças em relação ao leite humano.

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NEWBORN´S SEX AND LACTATION PHASE AFFECT THE PROTEIN ABUNDANCE OF HUMAN MILK

ABSTRACT

Human milk (HM) is a complete food that can meet all the nutritional and energetic demands of the newborn. In its composition, there is an expressive amount of bioactive proteins that have antimicrobial activity and that also plays important role in digestive and neurological development. However, in Brazil, the guidelines on the distribution of HM in milk banks are based on energy content. There are some studies in which HM macromolecules in HM were analyzed, showing differences in their composition between groups for some factors, such as the lactation phase and the newborn's sex. However, proteomic approaches relating to the impact of both these factors on HM composition are still scarce. We hypothesize that these factors affect the protein composition of HM, leading to the existence of specific and differentially abundant proteins, which may be relevant for the better development of the infant. Therefore, seeking to answer these questions, this study compares, through proteomic approaches (nano-LC/MS-MS), the differences presented in HM in the colostrum and mature phases, and between the sex of newborns. As a result, we obtained 142 identified proteins, 13 of which have not yet been identified in HM. Besides, we obtained 54 differentially abundant proteins between the lactation phases and 42 between the sex of the infant. These proteins were mainly related to metabolic and immune defense processes, suggesting that there are specific nutritional needs for females and males, and between the colostrum and mature phases. Based on our results, we point out the need to develop specific strategies for the distribution of HM in milk banks, in addition to changes in infant formulations to bring it closer to the composition of HM.

Keywords: human milk, comparative proteomics, newborn's sex, lactation phases

INTRODUCTION

Several newborns need special care while they are breastfed, like in the neonatal intensive care unit. However, we do not have guidelines on how to distribute the human milk (HM) that take into consideration some factors as the newborns sex or the lactation phase, which may impact on milk composition, and by so, they are nutritional characteristics. In Brazil, for example, the distribution of HM is based on energetic content [1].

The protein content in HM supplies a high source of amino acids and nitrogen, which are necessary for the growth and development of the baby. Furthermore, HM proteins show activity in nutrients digestion, protection against pathogens, and immune response [2]. In the literature, most of the studies with HM involve carbohydrates, lipids, and minerals, like calcium and sodium. Moreover, when analyzing newborn's sex as a factor that influences HM, we found only seven studies until 2020 [3-9], but in none of those, the proteins present were the focus, which shows the need for more studies.

Despite all the benefits shown in HM, it is very usual wean babies early. It is also common the need for hospitalizations for some babies, requiring nutritional strategies that support this newborn. In both situations, the importance of a better understanding of the HM protein profile is evident, searching for more efficient breastfeeding strategies.

Therefore, the objective of our study is to compare the protein content of HM in the colostrum and mature lactation stages, as well as the variation of the protein differences between newborns sex. We hypothesize that there are differentially abundant proteins for the groups. This study can lead to a better understanding of the nutritional profile of HM, assisting in specific nutritional strategies, being able to guide the distribution of milk to children. Moreover, this information can be applied to the improvement of infant formulas that best suit infants, especially concerning to protein content.

MATERIALS AND METHODS

Subjects and ethical aspects

This study was approved by the Human Research Ethics Committee in UFV (65105317.6.0000.5153), and informed consent was obtained from each mother before their participation. The samples of human milk (HM) were collected previously[10] from 39 breastfeeding mothers in colostrum and mature phases, separated according to infant sex. They were obtained from lactating women attended by the Lactation Support Program (PROLAC), in the city of Viçosa, Minas Gerais, Brazil. PROLAC is an extension program at the Federal University of Viçosa in partnership with Hospital São Sebastião and Human Milk Bank of Viçosa.

The samples were divided according to the newborn sex and the lactation phase, being subsequently stored at -80 °C until analysis in the current study. All mothers were in the same age group (15-39 years), and Supplementary Table 1 shows the information for each sample collected. So, to investigated the influence of the infant sex (female or male) and lactation phase (colostrum or mature phases) based on approached proteomics, we divided the samples into four groups: female colostrum (FC), male colostrum (MC), female mature (FM), and male mature (MM).

Sample preparation and quantification of soluble proteins

The HM samples were skimmed by centrifugation at 2,000 x *g* for 15 min to avoid interference by not soluble compounds in the proteomics analysis. After that, milk fat was removed, and the proteins of supernatant had the were separated according to Magalhães et al. [11], by precipitation overnight at -20 ºC in a cold acetone solution with 10% (v/v) trichloroacetic acid and 1 mM dithiothreitol (DTT) in a 1:6 ratio. After precipitation, we added cold acetone and centrifuged the samples four times at 20,100 x *g* for 30 min at 4 °C. The last centrifugation was done with 80% (v/v) ethanol and 1 mM DTT. The precipitated proteins were resuspended in 250 µL solubilization solution containing 2 M thiourea, 7 M urea, and 2% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS). The protein quantification of the samples was assessed by the Bradford method[12]. The absorbance analysis was performed in triplicates, at a wavelength of 595 nm.

Short-run electrophoresis

Pools of samples for each group (FC, MC, FM, and MM) were prepared to contain 60 µg of proteins each. The amount of sample needed to form the pools was based on the quantification of proteins, obtained by the Bradford method. Subsequently, three replicates of each pool of the groups were submitted in a short SDS-PAGE using 16% polyacrylamide gel (Mini-PROTEAN Tetra Cell; Bio-Rad). We used a constant current of 80 V until the samples reached 1 cm in the separation gel and then we ended the run. The gels were placed for 2 hours in fixative solution (40% methanol, 5% acetic acid) and then stained using Coomassie Brilliant Blue R-250 overnight (Bio-Rad)[13].

Analysis of samples by nano LC-MS/MS

For nano LC-MS / MS analysis of the samples, the entire lane on gels was excised, and the proteins digested with trypsin (Promega Trypsin Gold, Mass Spectrometry Grade) according to Shevchenko et al [14]. Each sample was analyzed using triplicates by the nano Acquity UPLC® (Waters, USA) at the Analytical Instrumentation Center of the Universidade de São Paulo (São Paulo-SP, Brazil). The peptides present in the replicates of each sample of the short-run were solubilized in 20 µL of an aqueous solution of formic acid 0.1% (degree of purity LCMS) and then 1 µL of the solution was analyzed in nano Acquity system. This system contains a model column with the nano Acquity UPLC® 2G-V / MTrap Symmetry® C18, 5 µm, 180 µm x 20 mm, which operates at a flow rate of 7 µL/min, for 3 minutes. The separation of the peptides occurred through a column of nano Acquity UPLC® BEH C18, 130Å, 1.7 µm, 100 µm x 100 mm, operating at flow rates of 0.3 µL/min. The mobile phase of the chromatographic process had as solvents water acidified with 0.1% formic acid (solvent A) and acetonitrile acidified with 0.1% formic acid (solvent B). The chromatographic mixture occurred according to the following schedule: 2% B for 1 minute; gradient of 2 to 30% B for 299 minutes; gradient of 30 to 85% B over 5 minutes; maintenance at 85% B for 5 minutes; gradient of 85 to 2% B over 5 minutes; and maintenance at 2% B for 5 minutes, totaling 320 minutes of chromatographic analysis.

The separated peptide samples were added on the mass spectrometer MAXIS 3G model (Bruker Daltonics, Germany), operating in online mode, with a

source of CaptiveSpray ionization. Peptide analysis was performed using an appropriate method for them (IE_GCF_01-02-2017), with a drying gas flow of 3 L/min, ionization source temperature of 150 ºC, and transmission voltage of 2 kV. After analysis, the data were converted into a mass list and submitted to the Mascot Daemon software (version 2.4.0, Matrix Science, London, UK) for confrontation against the *Homo sapiens* UniProt database. A human protein database was downloaded from UniProt on February/2020.

Criteria for proteins identification

To validate the Mascot results obtained by the nano LC-MS/MS analyses, we used the Scaffold software (version 4.10.0; http://www.proteomesoftware.com/). As confirmation parameters, we selected proteins and peptides with sequence identity probability above 95%, with at least 2 unique peptides and FDR (ratio of false positives) equal to zero. Proteins found at least in 2 replicates were considered identified. Therefore, we obtained a list of proteins present in the HM for each group and their scores, sequence coverage, and the number and sequence of detected peptides.

Bioinformatics Analysis

All proteins present in HM, in the colostrum and mature of mothers of female and male newborns, were analyzed using Gene Ontology (by the PANTHER, http://www.pantherdb.org/) and classified according to their molecular functions, protein class, and biological processes. Also, we performed a KEGG analysis (*https://www.genome.jp/kegg/pathway.html*) to evidence to which metabolic pathways the identified proteins belong to, and, using the software STRING 11.0 (https://string-db.org/) we analyzed the protein interaction networks for differentially abundant proteins in HM. The analysis of protein interaction networks was performed by the relationship of evidence between the interaction points presented in the network and the researched database, with reliability greater than 0.9. For analysis of protein interaction networks, we highlight proteins related to metabolic processes and defense pathways (immune system and antimicrobial functions).

Data analysis

Label-free quantitative proteomics was used to analyze changes in the level of abundance of HM proteins. For comparison between females and males, replicates of FC and FM formed female group (F) and replicates of MC and MM formed male group (M). The same strategy was applied to form the colostrum (Co) and mature (Ma) groups.

Multivariate Principal Component Analysis (PCA) was performed to evaluate protein abundances associated with lactations stage and sex conditions (phase and newborn sex). Therefore, to determine the differentially abundant proteins, we used a significance test (t-Student), where proteins with p-value < 0.05 were deemed statistically significant. The differential abundance proteins were demonstrated on a heatmap, showing the proteins that were up or down-regulated.

RESULTS

Characterization of the Human Milk Proteome

In this study, we identified 146 proteins present in human milk using nano LC-MS/MS (Supplementary Table 2). Of these, according to our bibliographic search 13 have not yet been identified in human milk (Table 1).

Table 1. Identified proteins previously unreported in human milk

UniProt	ID	Protein name	MW (kDa)[a]	pI[a]
P0DOY3	IGLC3_HUMAN	Immunoglobulin lambda constant 3	11.265	6.91
P01780	HV307_HUMAN	Immunoglobulin heavy variable 3-7	12.942	8.65
P06331	HV434_HUMAN	Immunoglobulin heavy variable 4-34	12.943	9.01
A0A075B6H7	KV37_HUMAN	Probable non-functional immunoglobulin kappa variable 3-7	12.784	5.14
A0A5H1ZRS9	A0A5H1ZRS9_HUMAN	Immunoglobulin kappa variable 2D-29	11.191	5.63
A0A0B4J2B5	A0A0B4J2B5_HUMAN	Immunoglobulin heavy variable 3/OR16-9	10.656	8.66
P01619	KV320_HUMAN	Immunoglobulin kappa variable 3-20	12.557	4.85
A0A4W8ZXM2	A0A4W8ZXM2_HUMAN	Immunoglobulin heavy variable 3-72	11.167	8.05
P01860	IGHG3_HUMAN	Immunoglobulin heavy constant gamma 3	41.325	8.23
P15531	NDKA_HUMAN	Nucleoside diphosphate kinase A	9.371	5.81
P53396	ACLY_HUMAN	ATP-citrate synthase	120.842	6.95
Q71U36	TBA1A_HUMAN	Tubulin alpha-1ª chain	57.73	4.94
P05546	HEP2_HUMAN	Heparin cofactor 2	57.73	4.94

a. theoretical value

The distributions of these proteins among the FC, FM, MC, and MM groups using the Venn diagram (Figure 1), evidenced differences between the mentioned groups in the presence and absence of some proteins, or different content for others proteins. Of the 146 proteins identified, 79 were common to all groups. Regarding with newborns sex, nine proteins were present only in females and 18 were present only in males. Moreover, when comparing the lactation phase, colostrum did not present unique proteins, while in mature, we identified 31 unique proteins.

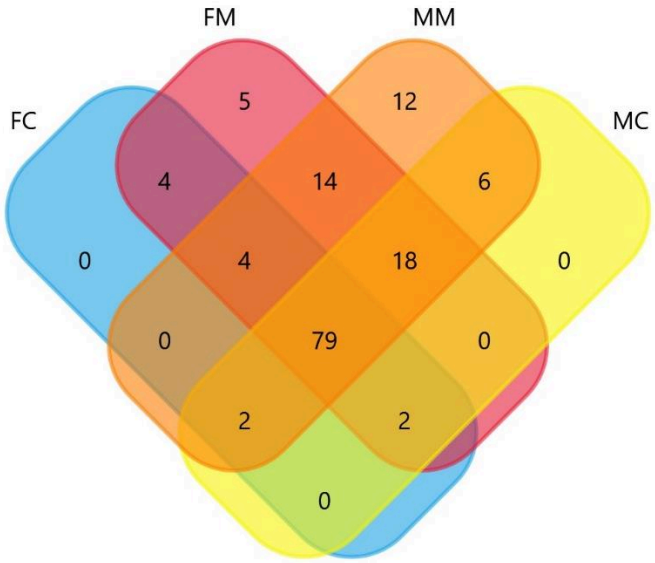


Figure 1. Distribution of proteins identified by nano LC-MS/MS in human milk between FC, FM, MM and MC groups by Venn Diagram (http://www.funrich.org/). FC, female colostrum; FM, female mature; MM, male mature; MC, male colostrum.

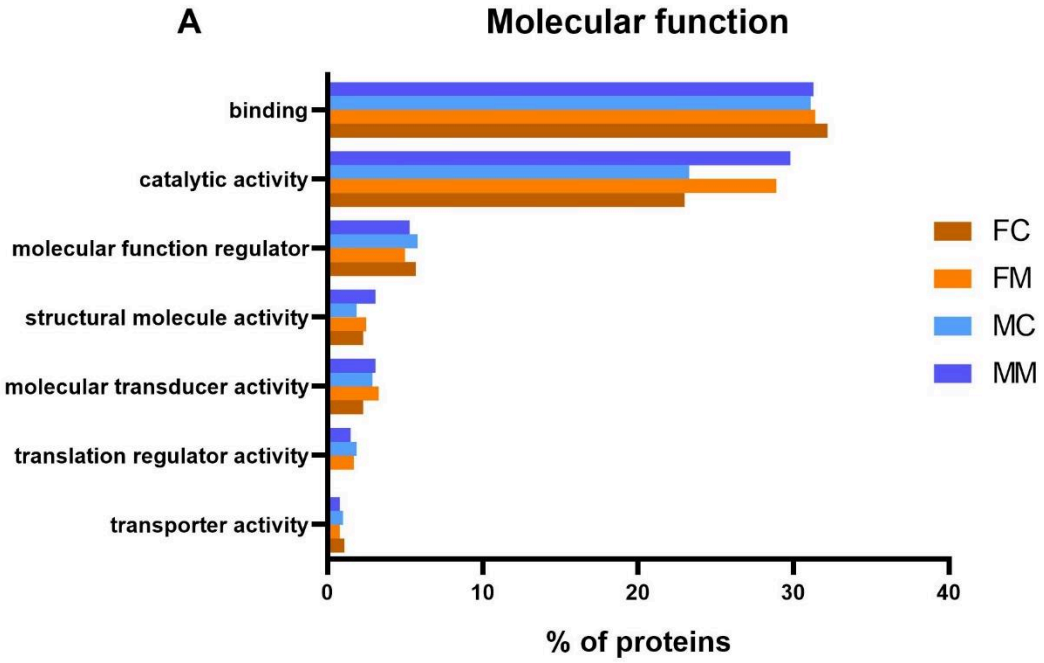
Functional classification of the Human Milk Proteome

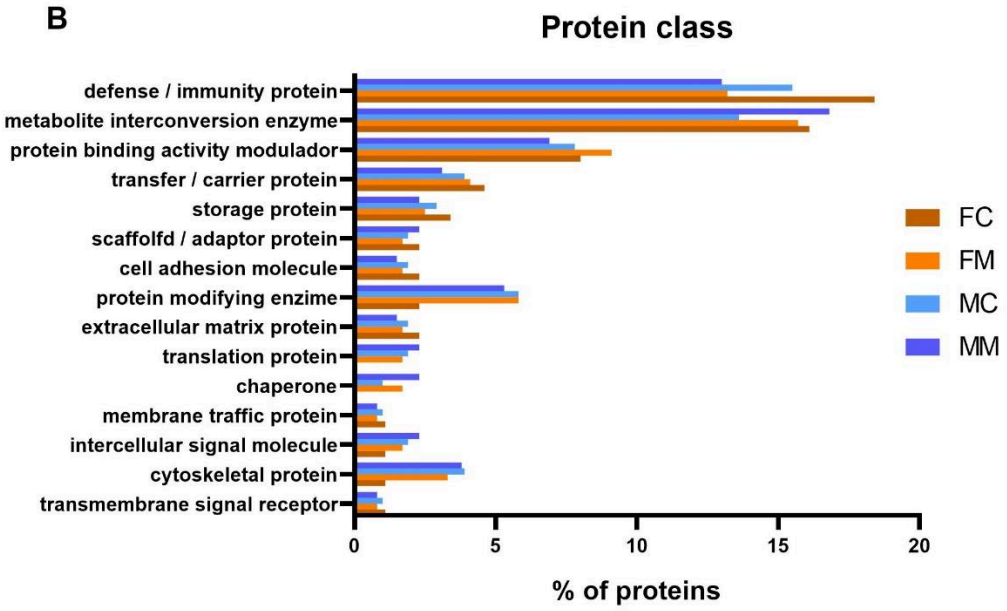
The 146 identified proteins were classified according to their molecular functions, protein class, and biological processes, as illustrated in Figure 2, using Gene Ontology. The four groups analyzed in this work - FC, FM, MC, MM - show distinct percentages within the different classifications.

As shown in Figure 2A, the main molecular functions of proteins are associated with binding (FC – 32.2%; FM – 31.4%; MC – 31.1% e MM – 31.3%) and catalytic activity (FC - 23%; FM – 28.9%; MC – 23.3% e MM – 29.8%).

Regarding to the protein classes (Figure 2B), the main ones are defense proteins (FC – 18.4%; FM – 13.2%; MC – 15.5% e MM - 13%) and metabolic interconversion enzymes (FC – 16.1%; FM – 15.7%; MC – 13.6% e MM – 16.8%).

For biological processes (Figure 2C), highlighted proteins involve cellular processes (FC – 35.6%; FM - 43%; MC – 36.9% e MM – 43.5%) and regulation (FC – 32.2%; FM – 29.8%; MC – 30.1% e MM - 29%).


A
Molecular function
binding
catalytic activity
molecular function regulator
structural molecule activity
molecular transducer activity
translation regulator activity
transporter activity
0
10
20
30
40
% of proteins
FC
FM
MC
MM


B
Protein class
defense / immunity protein
metabolite interconversion enzyme
protein binding activity modulador
transfer / carrier protein
storage protein
scaffolfd / adaptor protein
cell adhesion molecule
protein modifying enzime
extracellular matrix protein
translation protein
chaperone
membrane traffic protein
intercellular signal molecule
cytoskeletal protein
transmembrane signal receptor
0
5
10
15
20
% of proteins
FC
FM
MC
MM

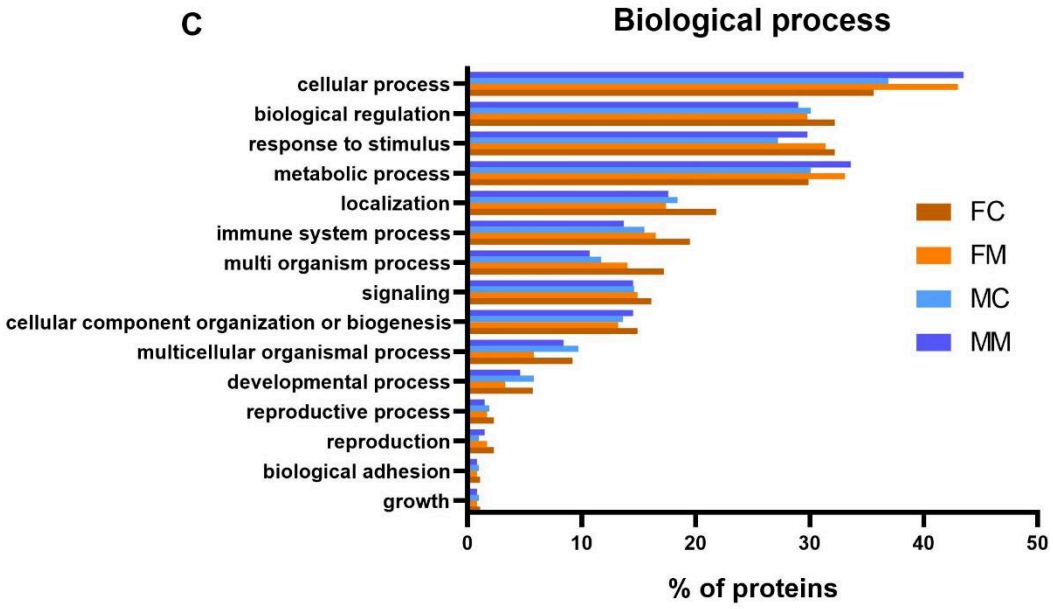


Figure 2. Functional classification of human milk proteins by Gene Ontology according to (A) molecular functions, (B) classes and (C) biological processes, identified by nano-LC/MS-MS. FC, female colostrum; FM, female mature; MC, male colostrum; MM, male mature.

KEGG pathway analysis

We performed KEGG pathway analysis of all identified proteins in human milk, comparing firstly colostrum and mature phase (Table 2) and then differences in HM between newborn sex (Table 3). The identified proteins participate in 26 KEGG pathways, with a distinction between phases and newborn sex.

Table 2. KEGG pathway analysis of proteins from colostrum and mature human milk

KEGG pathway name	Colostrum			Mature Milk		
	Count	%	Fold enrichment	Count	%	Fold enrichment
Metabolic pathways	-	-	-	24	16.7	1.5
Biosynthesis of antibiotics	6	5.3	3.2	14	9.7	5.2
Complement and coagulation cascades	10	8.8	15.6	12	8.3	13.6
PI3K-Akt signaling pathway	-	-	-	10	6.9	23
Carbon metabolismo	4	3.5	3.8	9	6.2	6.2
Phagosome	7	6.2	5	9	6.2	4.7
Protein processing in endoplasmic reticulum	5	4.4	3.2	9	6.2	4.2
Glycolysis / Gluconeogenesis	5	4.4	8	8	5.6	9.3
Biosynthesis of amino acids	4	3.5	6	8	5.6	8.7
Focal adhesion	6	5.3	3.1	7	4.9	2.7
Antigen processing and presentation	-	-	-	6	4.9	2.7
ECM-receptor interaction	5	4.4	6.2	6	4.2	5.4
Fat digestion and absorption	4	3.5	11	6	4.2	10
Legionellosis	4	3.5	8	5	3.5	7.2
Staphylococcus aureus infection	5	4.4	10	5	3.5	7.2
Pertussis	5	4.4	7.2	5	3.5	5.2
HIF-1 signaling pathway	5	4.4	5.6	5	3.5	4.1
Galactose metabolismo	3	2.7	10.7	4	2.8	10.4
Pathogenic *Escherichia coli* infection	3	2.7	6.3	4	2.8	6.1
PPAR signaling pathway	4	3.5	6.4	4	2.8	4.7
Prostate câncer	-	-	-	4	2.1	3.6
Vitamin digestion and absorption	-	-	-	3	2.1	10.7
Pentose phosphate pathway	-	-	-	3	2.1	8.1
Citrate cycle (TCA cycle)	-	-	-	3	2.1	7.8
Starch and sucrose metabolismo	-	-	-	3	2.1	7.1
Pyruvate metabolismo	-	-	-	3	2.1	5.9

PI3K, phosphatidylinositol 3' - kinase; ECM, extracellular matrix; HIF-1, Hypoxia-inducible factor 1; PPAR, Peroxisome proliferator-activated receptors. Count, number of proteins involved in the pathway; %, percentage of proteins; Fold enrichment, fold the ratio of enrichment of proteins.

Table 3. KEGG pathway analysis of proteins from female and male infants

KEGG pathway name	Females			Males		
	Count	%	Fold enrichment	Count	%	Fold enrichment
Metabolic pathways	21	16.7	1.5	23	17	6.5
Biosynthesis of antibiotics	12	9.5	5.1	12	9.6	5.1
Complement and coagulation cascades	12	9.5	15.5	11	8.1	13.2
PI3K-Akt signaling pathway	8	6.3	2.1	10	7.4	2.4
Carbon metabolismo	7	5.6	5.5	8	5.9	5.9
Phagosome	9	7.1	5.4	8	5.9	4.4
Protein processing in endoplasmic reticulum	6	4.8	3.2	9	6.7	4.4
Glycolysis / Gluconeogenesis	8	6.3	10.7	7	5.2	8.7
Biosynthesis of amino acids	7	5.6	8.7	7	5.2	8.1
Focal adhesion	7	5.6	3	7	5.2	2.8
Antigen processing and presentation	6	4.8	7.1	6	4.4	6.5
ECM-receptor interaction	6	4.8	6.2	6	4.4	5.7
Fat digestion and absorption	3	2.4	6.9	5	3.7	10.6
Legionellosis	4	3.2	6.6	5	3.7	7.7
Staphylococcus aureus infection	5	4	8.3	5	3.7	7.7
Pertussis	5	4	6	5	3.7	5.5
HIF-1 signaling pathway	5	4	4.7	5	3.7	4.3
Galactose metabolismo	4	3.2	11.9	4	3	11.1
Pathogenic *Escherichia coli* infection	4	3.2	7	4	3	6.5
PPAR signaling pathway	-	-	-	4	3	4.9
Prostate câncer	-	-	-	4	3	3.6
Vitamin digestion and absorption	-	-	-	3	2.2	11.3
Pentose phosphate pathway	3	2.4	9.2	3	2.2	8.6
Citrate cycle (TCA cycle)	-	-	-	3	2.2	8.3
Starch and sucrose metabolismo	3	2.4	8.1	3	2.2	7.5

PI3K, phosphatidylinositol 3' - kinase; ECM, extracellular matrix; HIF-1, Hypoxia-inducible factor 1; PPAR, Peroxisome proliferator-activated receptors. Count, number of proteins involved in the pathway; %, percentage of proteins; Fold enrichment, fold the ratio of enrichment of proteins.

The expression level of human milk proteins

Firstly, the multivariate principal component analysis (PCA), pointed to a good distribution of proteins between the groups Co x Ma and F x M. PC1 and PC2 could explain 63,18% of the variance in protein intensity indicating variations (Figure 3).

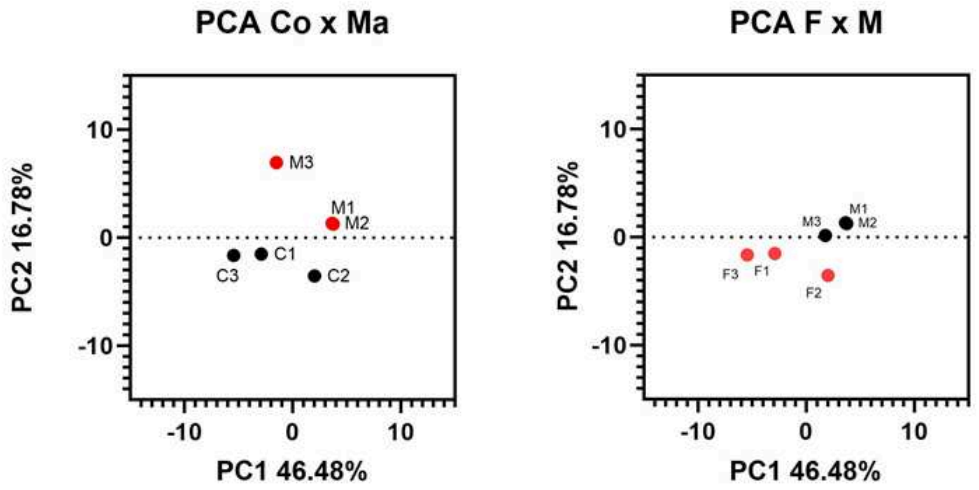


Figure 3. Multivariate Principal Component Analysis (PCA) of human milk. Co x Ma on the left and F x M on the right. CO, colostrum; MA, mature; F females; M, males.

The emPAI method present on the Scaffold software, followed by statistical evaluation by the t-Student with p-value <0.05, indicated the existence of differences in the abundance of the protein between Co x Ma and F x M. Therefore, we performed the analysis of the level of abundance of these proteins using a heatmap, obtaining the up and down-regulated proteins (Figures 4 and 5).

Furthermore, in this study, we also identified 31 proteins that were present in at least two of the three replicates performed to HM mature and were not present in any of the HM colostrum, being considered as exclusive of mature. Comparing the group F x M, there were nine proteins present in at least two of the three female replicates that were not present in the male replicates. Finally, there were 18 exclusive proteins on male replicates that were not present in female replicates. All these exclusive proteins were defined as up-regulated.

The heatmaps (Figure 4 for Co x Ma, and Figure 5 for F x M comparison) were constructed using only significantly different proteins that were present in both groups. Taking into consideration the significantly different proteins ($p<0.05$), illustrated in heatmaps, and the exclusive proteins for each group, we had 54 HM proteins that were differentially abundant for Co x Ma, and 42 for F x M. These differentially abundant proteins in HM are present in the Supplementary Tables 3 and 4.

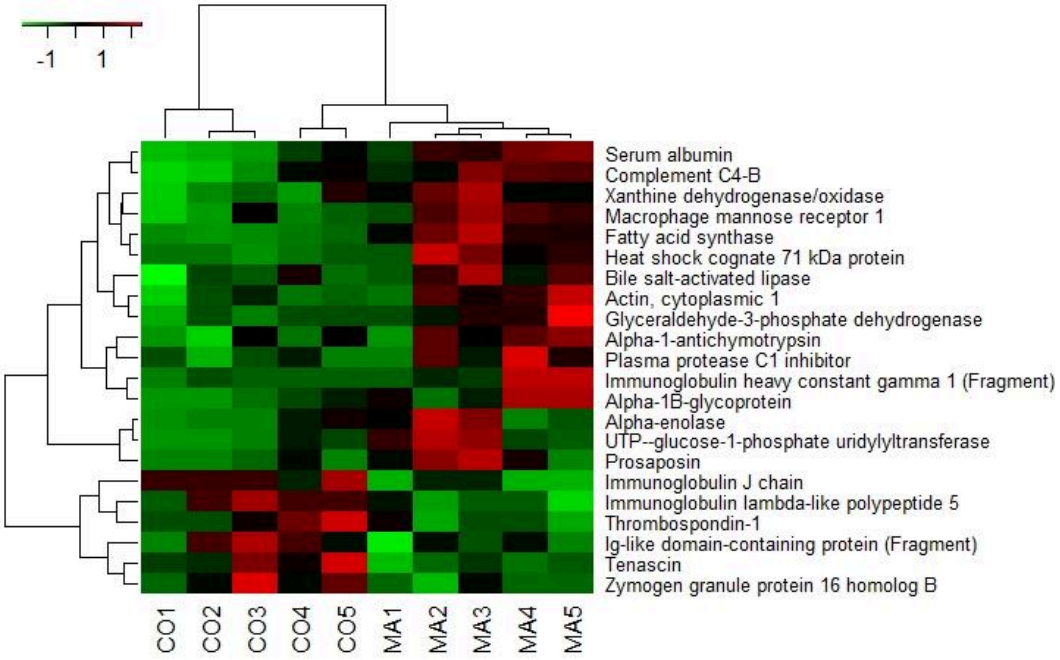


Figure 4. Heatmap for proteins with differences in abundance on Co x Ma. The columns represent the groups and the lines the proteins, where the red colors had higher abundant levels, and green had lower. Co, colostrum; Ma, mature.

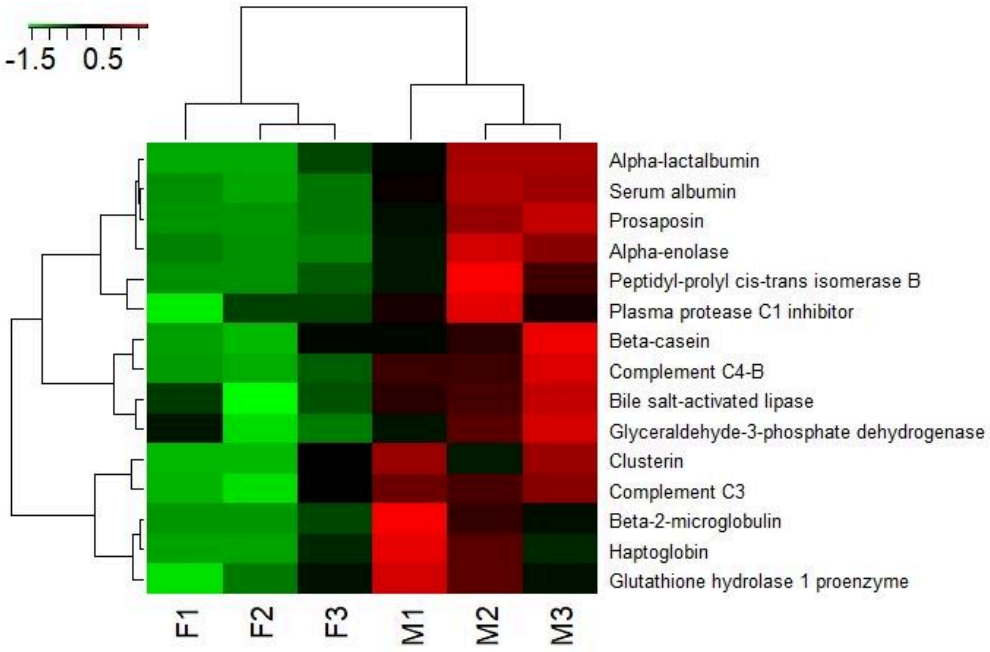


Figure 5. Heatmap for proteins with differences in abundance on F x M. The columns represent the groups and the lines the proteins, where the red colors had higher abundant levels, and green had lower. F females; M, males.

Functional classification of exclusive proteins

We also evaluated the functional classification of proteins that were exclusive between the groups. These unique proteins were classified according to their molecular functions, protein class, and biological processes, as shown in Figures 6 and 7.

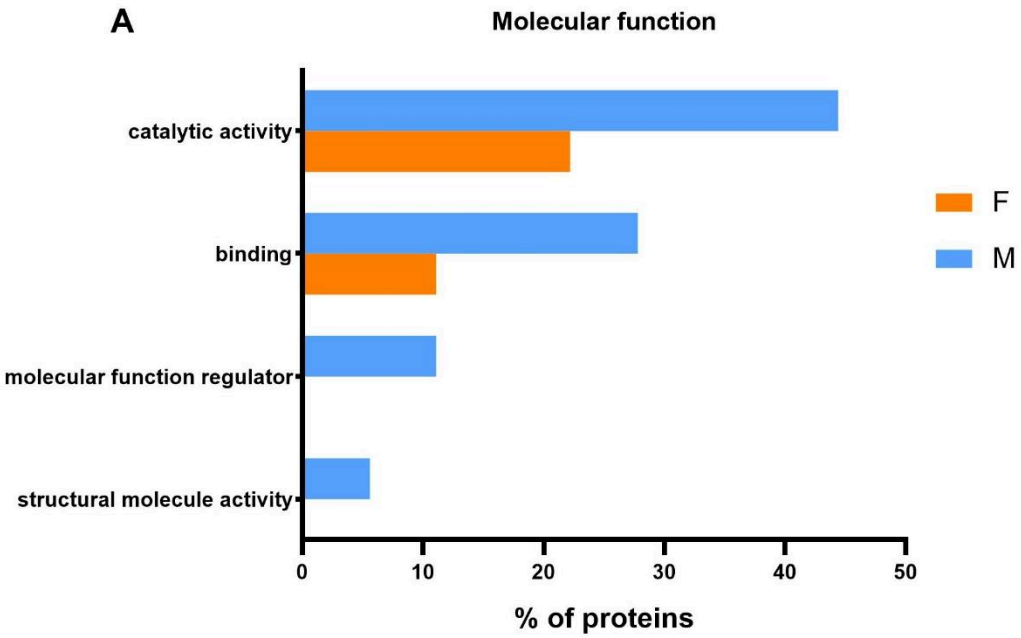
For the classification by molecular functions (Figure 6A), group M proteins demonstrated greater catalytic activity (M - 44.4% and F - 22.2%) and binding (M - 27.8% and F - 11, 1%). Structural activity (M - 5.6%) and molecular regulation (M - 11.1%) were found only in M group.

As shown in the classes of proteins (Figure 6B), in the group M there were more proteins related to metabolic interconversion (M - 16.7% and F - 11.1%) while for group F proteins from extracellular matrices (M - 5.6% and F - 11.1%) and defense (M - 5.6% and F - 11.1%) were higher. Furthermore, some classes of proteins were presented only in one group. The protein classes modulating binding

proteins and carriers were present in group F while chaperone, translation, folding activity, among others, were present in group M.

For cellular (M - 50% and F - 33.3%), metabolic (M - 44.4% and F - 22.2%), and control processes (M - 22.2% and F - 11.1%), and also for biogenesis (M - 22.2% and F - 11.1%), proteins were higher in group M, while responses to stimulus (M - 22.2% and F - 11.1%) and multiple organism processes (M - 16.7% and F - 33.3%) were higher for group F (Figure 6C). Still, there are immune system proteins only in group F, and development only in M.

When evaluating the colostrum and mature phase, as colostrum did not present any exclusive protein, we evaluated only the mature phase, where we found 31 proteins (Figure 7).



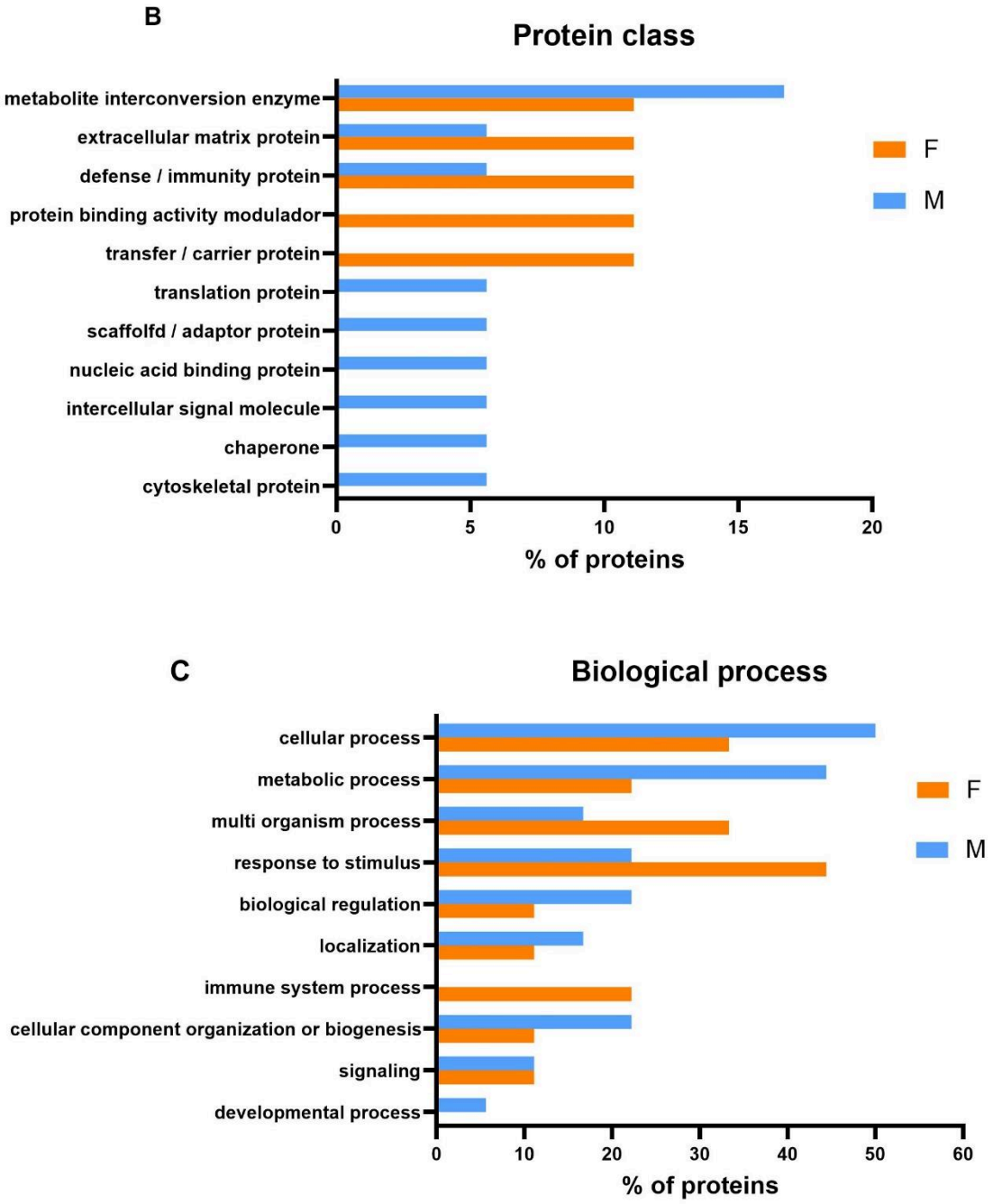
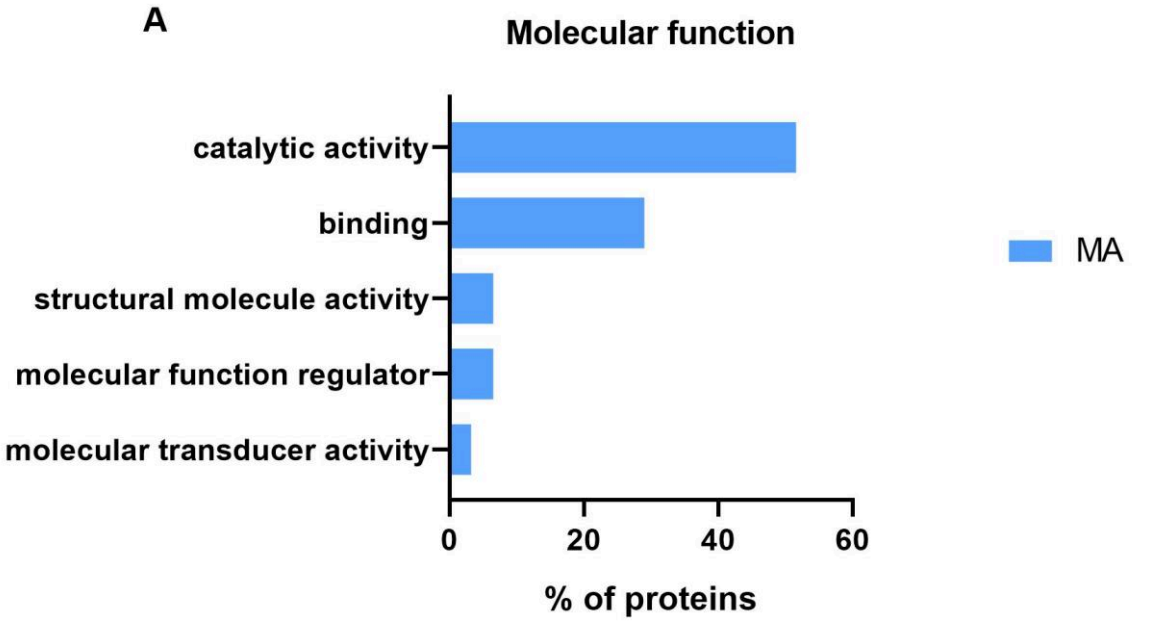
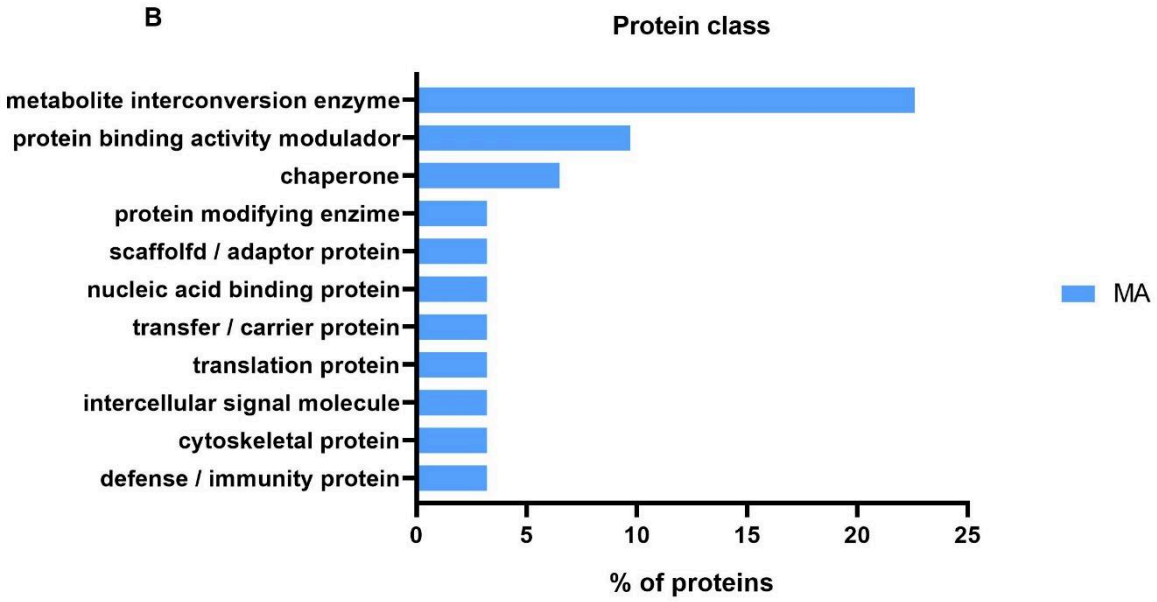


Figure 6. Functional classification of human milk differential proteins between groups F and M by Gene Ontology according to (A) molecular functions, (B) protein classes and (C) biological processes, identified by nano-LC/MS-MS. F, females; M, males.


A
Molecular function
catalytic activity
binding
structural molecule activity
molecular function regulator
molecular transducer activity
0
20
40
60
% of proteins
MA


B
Protein class
metabolite interconversion enzyme
protein binding activity modulador
chaperone
protein modifying enzime
scaffolfd / adaptor protein
nucleic acid binding protein
transfer / carrier protein
translation protein
intercellular signal molecule
cytoskeletal protein
defense / immunity protein
0
5
10
15
20
25
% of proteins
MA

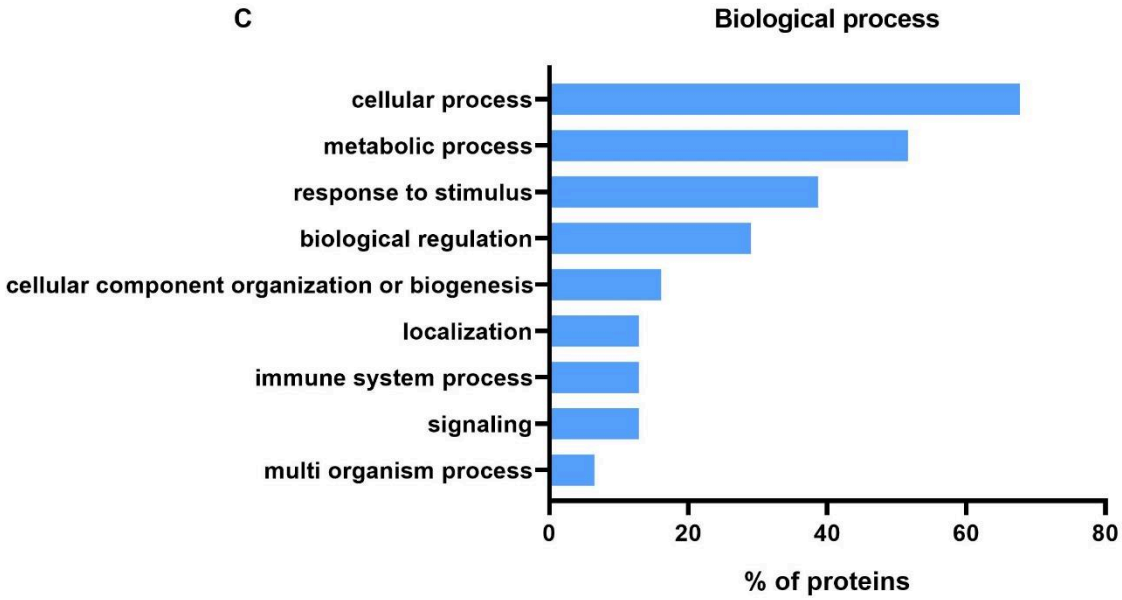


Figure 7. Functional classification of human milk differential proteins in group Ma by Gene Ontology regarding (A) molecular functions, (B) protein classes and (C) biological processes, identified by nano-LC/MS-MS. Ma, mature human milk.

Analysis of protein interaction networks

The interaction networks between differentially abundant proteins identified in human milk are illustrated in the figures below. For each group (F x M and Co x Ma) we made comparisons with proteins related to the defense pathways (immune system and antimicrobial functions) and classifications also regarding proteins related to metabolism.

Figure 8 shows the interaction network for proteins related to the defense system in HM between females and males. We were able to observe that in the group of females there were a greater number of proteins that shown this biological function, corroborating with the previous analyzes already demonstrated. While in Figure 9 the classification was related to proteins involved in metabolic pathways and shown a higher number in the group of males.

Regarding the interaction network according to the lactation phase, analyzing the defense system (Figure 10) in colostrum, there were a greater number of proteins that shown defense activity. For proteins related to metabolism (Figure 11), the

mature group had a higher number of proteins, indicating in this phase a greater involvement for the infant's energy production.

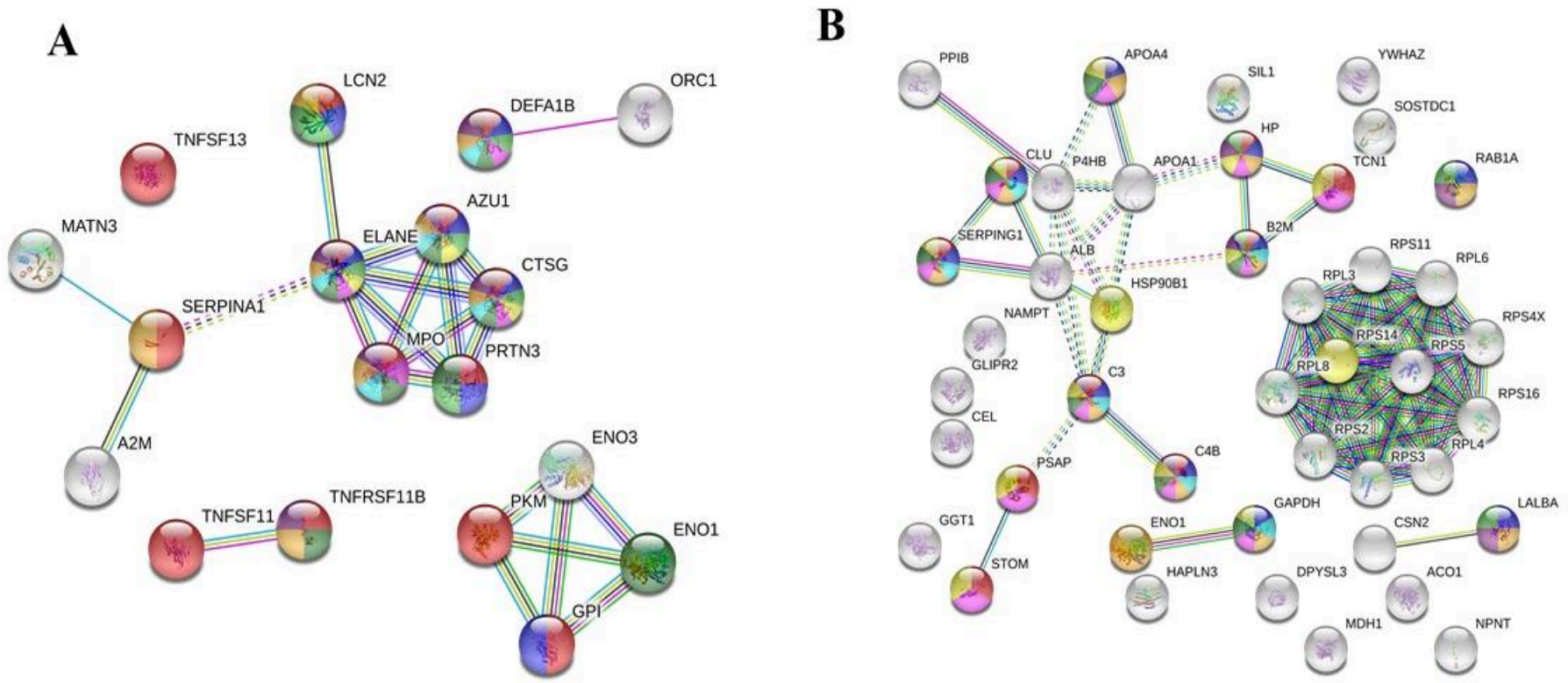


Figure 8. Interaction networks of differentially abundant proteins in human milk for females and males. Colored nodes are related to the defense system. The network was obtained through the software STRING 11.0. (A) protein interaction for females, (B) protein interaction for males.

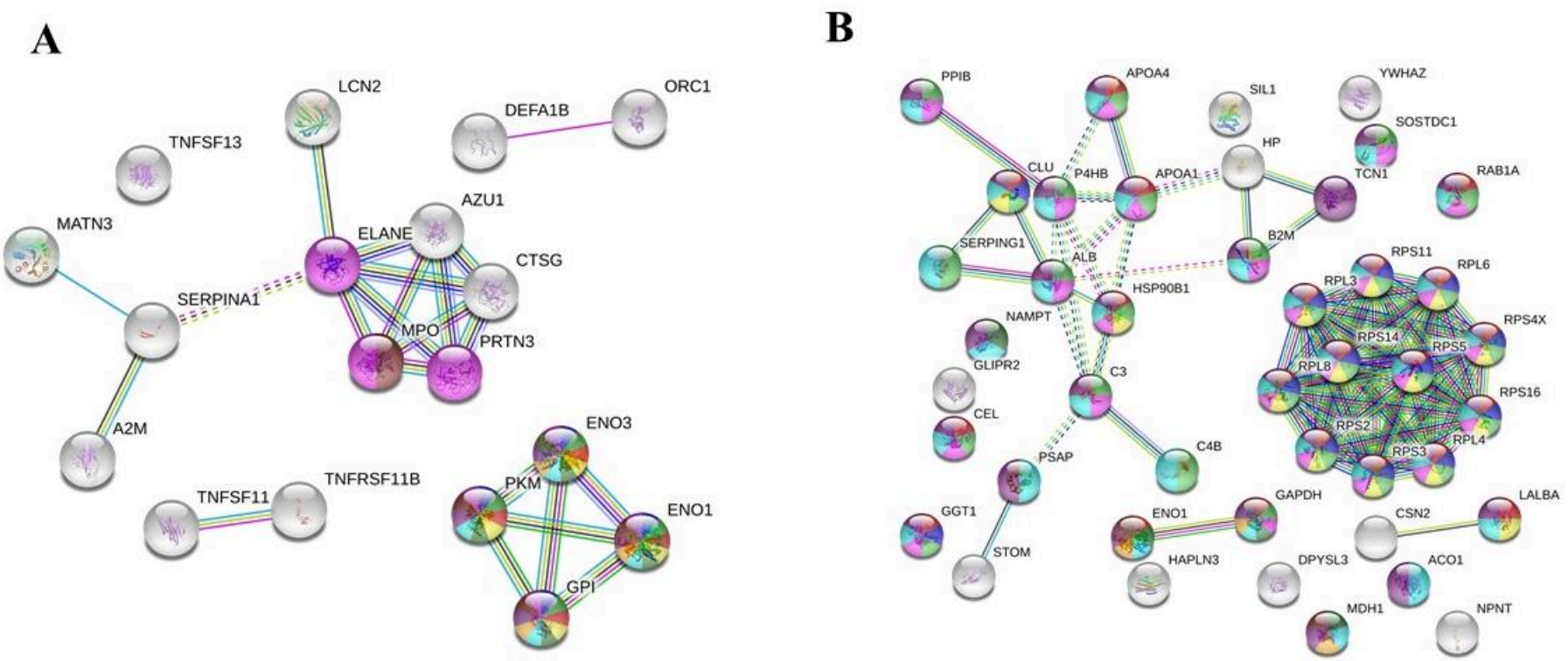


Figure 9. Interaction networks of differentially abundant proteins in human milk for females and males. Colored nodes are related to metabolism. The network was obtained through the software STRING 11.0. (A) protein interaction for females, (B) protein interaction for males.

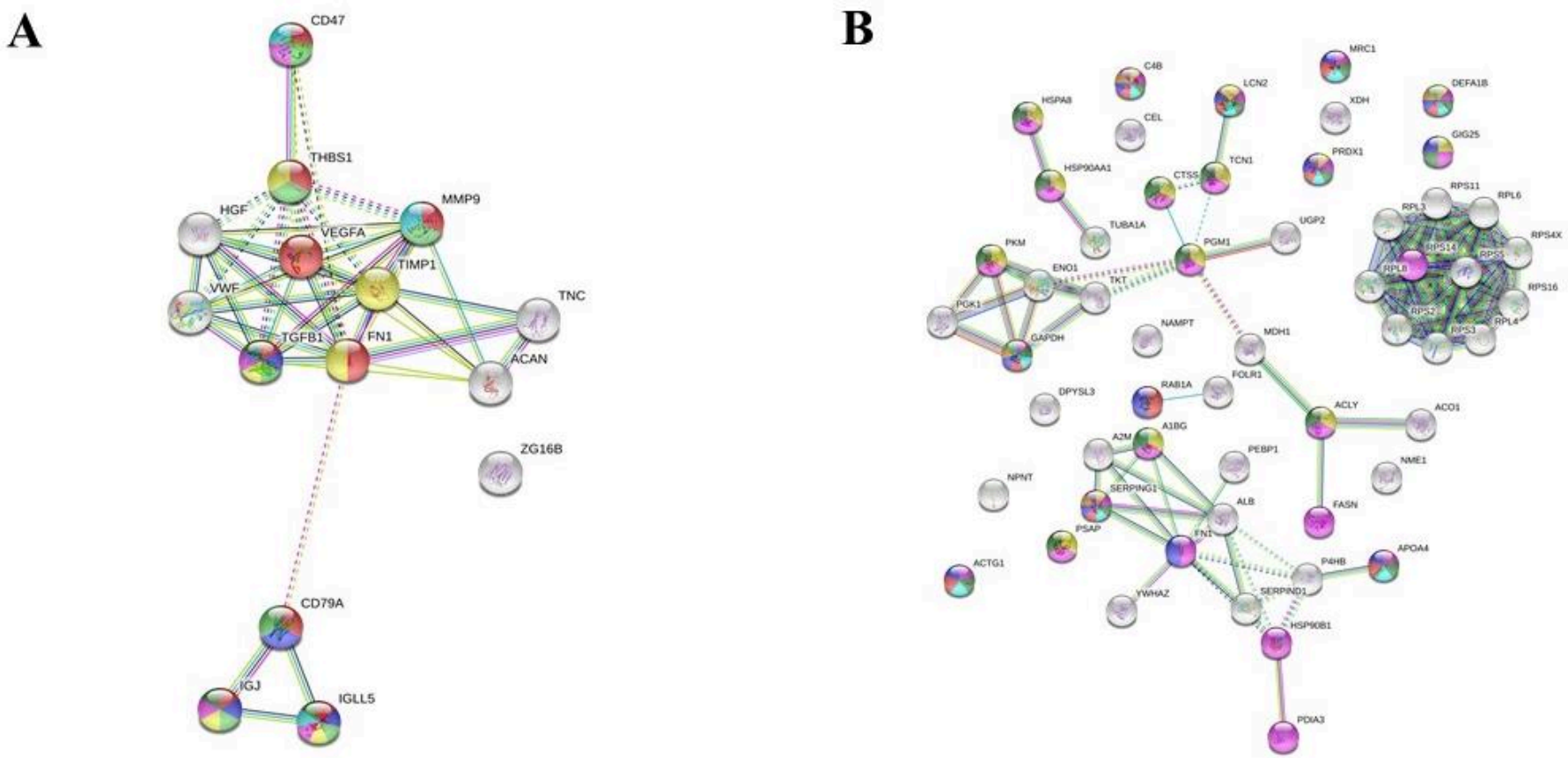


Figure 10. Interaction networks of differentially abundant proteins in human milk for colostrum and mature. Colored nodes are related to the defense system. The network was obtained through the software STRING 11.0. (A) protein interaction for colostrum, (B) protein interaction for mature.

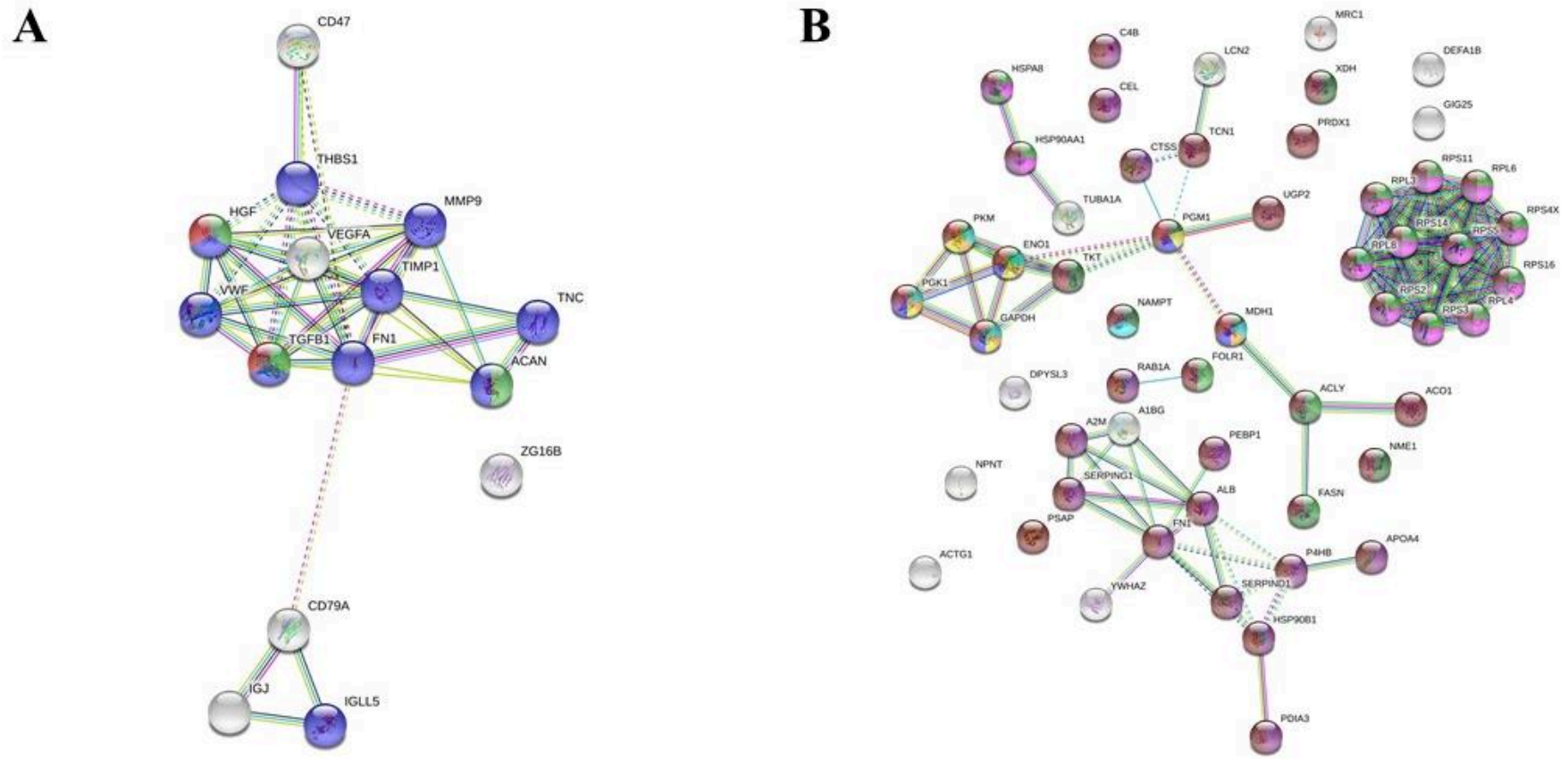


Figure 11. Interaction networks of differentially abundant proteins in human milk for colostrum and mature. Colored nodes are related to metabolism. The network was obtained through the software STRING 11.0. (A) protein interaction for colostrum, (B) protein interaction for mature.

DISCUSSION

Total proteome of HM

In this study, we used nano LC-MS/MS analysis to characterize the total human milk (HM) proteome and also the differential proteome of the colostrum and mature phases, as well as newborn sex that was receiving the HM. In our comparative proteome analyzes, we identified differentially abundant proteins in each group, which were confirmed by statistical tests, and we also identified unique proteins that were present only in specific analyzed groups. These proteins raise questions about the biological functions they perform in the HM**.**

The 146 proteins identified in the total proteome of the HM (Supplementary Table 1) were classified as belonging to groups with varied biological functions, as well as their participation in metabolic pathways according to the KEGG tool (Tables 2 and 3). Due to its nutritional, energetic and development roles[2,15,16], confirmed by the differences between infants fed with HM or formula[17,18], it seems that the HM protein composition may have a fundamental impact on the infant growth and general development. Moreover, considering that evolution aims typically to avoid the unnecessary use of resources, it can be assumed that most of these proteins can have their function preserved when used by the infant´s body, since they act regardless of the infants' age or sex. This fact was evidenced in our results because 54.1% of the 146 proteins identified in HM were detected in all evaluated groups. This behavior was already suggested by early studies that shown the impact of HM in the immunologic defense development in newborns.

We identified 13 proteins that had not yet been identified in human milk, according to our bibliographic search. These proteins are mainly associated with immune activity (Table 1).

Influence of lactation and infant sex on human milk

There are reports in the literature that suggest that males are less protected than females during growth and development, and are more likely to be affected by environmental stresses[19]. In our results, comparing HM according to the infant sex, we confirmed that there is a higher number of proteins involved with the immune system present in the female's group than there is in the male's group. This fact also

can reaffirm the idea that since birth, the female is already being prepared for a future stage of pregnancy and lactation, with the need for a complete defense system, which may begin with a greater abundance of defense proteins in the milk of the female infants. Likewise, concerning the lactation phases, in the HM colostrum there were also more proteins related to the immune system, whereas, in the mature phase, the proteins related to metabolism and energy production were increased.

HM also has a variety of antimicrobial proteins (usually resistant to intestinal protein) that work both, to protect the lactating mammary gland and to protect the infant, since its immune system is still in development[20]. It was possible to identify in the total proteome of HM, several proteins with this function, among them, beta-2-microglobulin, neutrophil defensin 1, neutrophil gelatinase-associated lipocalin, complement C3, monocyte differentiation antigen CD14 e haptoglobin. The study of groups of proteins with antimicrobial activity can assist in improving infant formulas, being a critical complement in the diet of infants who use it. In this way, infant formulations could be closer to the composition and benefits present in HM.

Differentially abundant proteins in human milk have immune and metabolism functions

Besides the characterization of the general HM proteome, we also identified specific proteins that were differentially abundant between the lactation phases and the infant sex. The well-know protein clusterin was one of them, being more present in the HM of the male group (Figure 5). Considering its various functions, such as chaperone activity, apoptosis, and the immune system, Hettinga[21], suggested that clusterin may also be related to the protection of the intestine, maintaining its homeostasis and regeneration. They indicated that it might have similar functions as *milk fat globule–EGF factor 8*, a crucial factor for enhancing enterocyte migration in vitro as well as in vivo, assisting in homeostasis. The literature suggested that males consume a higher volume of milk compared to females[22], therefore, clusterin's higher abundance may act aiding in the protection of the male's digestive system.

Alpha-lactoalbumin and serum albumin also play important roles in the immune system and were identified as differentially abundant. Alpha-lactoalbumin revealed more abundance for males (Figure 5), and serum albumin more abundance for males and in the mature phase (Figure 4 and 5).

The proteins complement C3, glyceraldehyde 3 phosphate dehydrogenase, alpha-enolase, bile salt activated lipase, and prosaposin were also identified as differentially abundant in the males group (Figure 5). These proteins act in metabolic processes, participating in important pathways, as glycolytic and beta-oxidation ways. Considering that there is evidence that the male fetus grows faster than the female fetus [23], and this continues throughout life, where usually females are smaller than males[24], this may suggest that, also since birth, the male infant may be being prepared for faster energy absorption and increased development.

As examples, alpha-enolase is known to participate in the glycolytic pathway, and it is also related to muscle growth and development[25], while Bile salt-activated lipase plays an essential role in the digestion and absorption of lipids[26]. An effective efficiency of lipids is important for the use of energy, and also for the optimization of human growth and development. Bile salt-activated lipase is a key enzyme for digesting various substrates, such as triglyceride, phospholipids, vitamins[27]. Both these proteins influence on how the organism will grow and were exhibited with more abundance for males, reinforcing the possibility that they may have their function preserved in the infant body.

Also, for the exclusive proteins identified, 5 of the 9 proteins present for the female group are related to immunological processes, being the main function present for this group (myeloperoxidase, matrilin 3, heavy constant range of immunoglobulin gamma 3, defense of neutrophils 1, alpha 2 macroglobulin, and lipocalin associated with neutrophil gelatinase). While for males, the main functions were related to the metabolism and transport of proteins and lipids (nucleotide exchange factor S1L1, apolipoprotein A I, transcobalamin I, nicotinamide phosphoribosyltransferase, ras-related protein rab I A e apolipoprotein A-IV). The transport activity of these proteins may be related to the supply of amino acids and nutrients to newborns, and in our study, they were present only in the male group.

Differentially abundant proteins from human milk suggest specific nutritional needs for females and males, and between the colostrum and mature phases

Complementing the results already presented, we did an interaction network for differentially abundant proteins, and the group of females presented more defense-related proteins (Figure 8A) compared to males (Figure 8B). In these

interactions, we highlight the alpha-enolase protein, which is up-regulated for both, mature milk and the female group. Although it is not directly related to the immune system, it plays important role in the metabolism of females, participating in the pathways of glycogenesis, gluconeogenesis, and amino acid biosynthesis.

The interactions of differentially abundant proteins in the colostrum phase (Figure 10A), evaluating defense functions, gave rise to a large cluster involving most of the proteins present, signaling an essential role in the immune system in this lactation phase. Differential proteins in the mature phase were intensely present in the interaction network when the metabolic processes were evaluated (Figure 11B).

Finally, the differentially abundant proteins and exclusive proteins in HM, analyzing the infant sex and lactation phase, revealed a significant difference in terms of their functional and biological classification. These proteins play varied and important roles in the protection of the intestine, interaction with the membrane of pathogens, cellular apoptosis, transport of amino acids (previously mentioned). This variety of biological functions corroborating our hypothesis of the importance and influence that these factors have on the HM. In a recent study in which twins of the same sex and opposite sex who were breastfed only with HM, was demonstrated that twins of different sex grew less than same-sex ones[28].This result reinforces our idea, demonstrating that males and females have specific nutritional needs for their growth and development. Since milk is the first food that a human being has contact with and considering its importance in the development[29], based on our study, we suggest that guidelines related to the newborn sex should be considered for the distribution of HM. Thus, the required nutritional needs could be more appropriate for the infant.

CONCLUSION

In this study, we did the characterization of the HM proteome in the colostrum and mature lactation phases, as well as the variation of these differences between newborn sex. We found differentially abundant proteins between the groups, which have distinct biological functions. These proteins can play important roles in the development of the newborn. The continuity of this study can be applied to evaluate children who use the milk bank, analyzing whether they are meeting their needs in the best way. Moreover, our study can be used to the improvement of infant formulas

that best suit infants, especially concerning protein content, based on these differentially abundant proteins.

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Supplementary Table 1. Data from milk samples collected in this study.

Sample	Age of the mother	Newborn's sex	Colostrum	Mature
			Days after partum	Days after partum
1	20	F		21
2	29	M	6	33
3	21	F	4	20
4	19	M	6	
5	20	M	6	20
6	18	M		19
7	37	F	5	
8	25	M	5	
9	31	M	6	
10	40	F	6	
11	33	M		15
12	39	M		21
13	39	F	6	20
14	22	F		22
15	15	F	6	21
16	36	M	3	18
17	35	F	5	
18	35	F		20

F, female; M, male.

Supplementary Table 2. Identified proteins in the human milk using nano LC MS/MS

UniProt	ID	Protein name	MW (kDa)[a]	pI[a]	Peptide sequence	Probability of peptide identity (%)	Coverage (%)
P07996	TSP1_HUMAN	Thrombospondin-1	129.381	4.70	GGVNDNFQGVLQNVR	98,0	5
					GTSQNDPNWVVR	100	
					FVFGTTPEDILR	97.6	
					TIVTTLQDSIR	99.9	
					GTLLALER	99.2	
P19835	CEL_HUMAN	Bile salt-activated lipase	79.667	5.08	ALENPQPHPGWQGTLK	100	28
					AMIAYWTNFAK		
					EAQMPAVIRF		
					GIPFAAPTK		
					KVTEEDFYK		
					LGAVYTEGGFVEGVNK		
					LGLLGDSVDIFK		
					LVSEFTITK		
					NPLFWAK		
					TTFDVYTESWAQDPSQENK		
					TYAYLFSHPSR		
					VGCPVGDAAR		
					AISQSGVALSPWVIQK		
					ALTLAYK		
					DQHMAIAWVK		
					VGPLGFLSTGDANLPGNYGLR		
					MPVYPK		
					GNVIVVTFNYR		
P02787	TRFE_HUMAN	Serotransferrin	77.074	6.70	CSTSSLLEACTFR	100	38
					DSGFQMNQLR		
					EGYYGYTGAFR		

					MYLGYEYVTAIR		
					SASDLTWDNLK		
					SVIPSDGPSVACVKK		
					ADRDQYELLCLDNTR		
					GDVAFVK		
					IECVSAETTEDCIAK		
					LCMGSGLNLCEPNNK		
					NLNEKDYELLCLDGTR		
					DLLFRDDTVCLAK		
					EFQLFSSPHGK		
					KASYLDCIR		
					YLGEEYVK		
					HSTIFENLANK		
					FDEFFSEGCAPGSK		
					EGTCPEAPTDECKPVK		
					KPVEEYANCHLAR		
					WCAVSEHEATK		
					TAGWNIPMGLLYNK		
P01011	AACT_HUMAN	Alpha-1-antichymotrypsin	47.653	5.32	ADLSGITGAR	100	37
					EQLSLLDR		
					ITLLSALVETR		
					KLINDYVK		
					AVLDVFEEGTEASAATAVK		
					MEEVEAMLLPETLKR		
					AKWEMPFDPQDTHQSR		
					DSLEFR		
					EIGELYLPK		
					NLAVSQVVHK		
					GKITDLIK		
					LYGSEAFATDFQDSAAAK		
					DEELSCTVVELK		
P01833	PIGR_HUMAN		83.283	5.59	ADAAPDEKVLDSGFR	100	39

		Polymeric immunoglobulin receptor			AFVNCDENSR RAPAFEGR CGLGINSR GVAGGSVAVLCPYNR IIEGEPNLK NADLQVLKPEPELVYEDLR VLDSGFREIENK VPCHFPCK VYTVDLGR DGSFSVVITGLR WNNTGCQALPSQDEGPSK FSSYEK ADEGWYWCGVK APAFEGR CPLLVDSEGWVK GGCITLISSEGYVSSK GSVTFHCALGPEVANVAK ILLNPQDK KYWCR LSDAGQYLCQAGDDSNSNK QSSGENCDVVVNTLGKR TVTINCPFK LVSLTLNLVTR		
P01871	IGHM_HUMAN	Immunoglobulin heavy constant mu	49.438	6.35	LICQATGFSPR NVPLPVIAELPPK QVGSGVTTDQVQAEAK VSVFVPPR YVTSAPMPEPQAPGR DVMQGTDEHVVCK GVALHRPDVYLLPPAR VTSTLTIK YAATSQVLLPSK	100	40

					DGFFGNPR		
					EQLNLR		
					FTCTVTHTDLPSPLK		
					QIQVSWLR		
					VFAIPPSFASIFLTK		
					GFPSVLR		
					GQPLSPEK		
P10451	OSTP_HUMAN	Osteopontin	35.422	4.35	AIPVAQDLNAPSDWDSR	100	18
					YPDAVATWLNPDPSQK	100	
					GDSVVYGLR	99.9	
					ISHELDSASSEVN	100	
P05155	IC1_HUMAN	Plasma protease C1 inhibitor	59.493	5.97	FQPTLLTLPR	99.6	14
					TNLESILSYPK	100	
					DFTCVHQALK	100	
					GVTSVSQIFHSPDLAIR	100	
					LLDSLPSDTR	99.8	
					LEDMEQALSPSVFK	100	
P19440	GGT1_HUMAN	Glutathione hydrolase 1 proenzyme	61.41	6.65	GGLSVAVPGEIR	99.8	9
					GLAAALENKR	100	
					LFQPSIQLAR	99.8	
					IVEAFR	100	
					NIDQAVTAALETR	100	
P06858	LIPL_HUMAN	Lipoprotein lipase	53.163	8.23	LVAALYK	100	22
					LVGQDVAR		
					GKAPAVFVK		
					DFIDIESK		
					GLGDVDQLVK		
					ITGLDPAGPNFEYAEAPSR		
					AQEHYPVSAGYTK		

					LSPDDADFVDVLHTFTR		
					EPDSNVIVVDWLSR		
P04406	G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	36.053	8.58	IISNASCTTNCLAPLAK	100	30
					LISWYDNEFGYSNR		
					LTGMAFR		
					LVINGNPITIFQERDPSK		
					VIPELNGK		
					VGVNGFGR		
					GALQNIIPASTGAAK		
					VPTANVSVVDLTCR		
P45877	PPIC_HUMAN	Peptidyl-prolyl cis-trans isomerase C	22.763	8.48	TVENFVALATGEK	97.8	10
					IVIGLFGK	99.8	
P61769	B2MG_HUMAN	Beta-2-microglobulin	11.732	6.06	IQVYSR	99.8	22
					VEHSDLSFSK	100	
					VNHVTLSQPK	99.8	
P07339	CATD_HUMAN	Cathepsin D	44.238	6.10	VSTLPAITLK	99.7	14
					AIGAVPLIQGEYMIPCEK	100	
					FDGILGMAYPR	100	
					LSPEDYTLK	99.8	
					VGFAEAAR	99.8	
Q99541	PLIN2_HUMAN	Perilipin-2	48.075	6.36	DAVTTTVTGAK	100	24
					DSVASTITGVMDK		
					EVSDSLLTSSK		
					SVVSGSINTVLGSR		
					TITSVAMTSALPIIQK		
					LEPQIAVANTYACK		
					AYQQALSR		
					LPILNQPSTQIVANAK		
P36222	CH3L1_HUMAN	Chitinase-3-like protein 1	42.627	8.65	LVMGIPTFGR	100	31
					EAGTLAYYEICDFLR	100	

					QLLLSAALSAGK	100	
					TLLSVGGWNFGSQR	100	
					GNQWVGYDDQESVK	100	
					VTIDSSYDIAK	100	
					ILGQQVPYATK	99.8	
					FPLTNAIK	99.8	
					SFTLASSETGVGAPISGPGIPGR	100	
P02788	TRFL_HUMAN	Lactotransferrin	77.970	8.50	ADAVTLDGGFIYEAGLAPYK	100	85
					CAFSSQEPYFSYSGAFK		
					CFQWQR		
					CGLVPVLAENYK		
					CLAENAGDVAFVK		
					CLRDGAGDVAFIR		
					CSTSPLLEACEFLR		
					DEYELLCPDNTR		
					DGAGDVAFIR		
					DLLFKDSAIGFSR		
					DSPIQCIQAIAENR		
					DVTVLQNTDGNNNEAWAK		
					EDAIWNLLR		
					ESTVFEDLSDEAER		
					FCLFQSETK		
					FDEYFSQSCAPGSDPR		
					FFSASCVPGADK		
					FKDCHLAR		
					FQLFGSPSGQK		
					GEADAMSLDGGYVYTAGK		
					GGSFQLNELQGLK		
					GPPVSCIK		
					GQFPNLCR		
					IDSGLYLGSGYFTAIQNLR		
					KCSTSPLLEACEFLR		

					KPVDKFK		
					KSCHTAVDR		
					KSEEEVAAR		
					LADFALLCLDGK		
					LKQVLLHQQAK		
					LRPVAAEVYGTER		
					MDKVER		
					NGSDCPDKFCLFQSETK		
					NLLFNDNTECLAR		
					QVLLHQQAK		
					RDSPIQCIQAIAENR		
					RKPVTEAR		
					SCHLAMAPNHAVVSR		
					SCHTGLRR		
					SDTSLTWNSVK		
					SDTSLTWNSVKGK		
					SNLCALCIGDEQGENK		
					SQQSSDPDPNCVDRPVEGYLAVAVVR		
					SVNGKEDAIWNLLR		
					SVQWCAVSQPEATK		
					THYYAVAVVK		
					VPPRIDSGLYLGSGYFTAIQNLR		
					VPSHAVVAR		
					VRGPPVSCIK		
					VVWCAVGEQELR		
					YLGPQYVAGITNLK		
					YYGYTGAFR		
					MDKVER		
					SDTSLTWNSVKGK		
P47710	CASA1_HUMAN	Alpha-S1-casein	21.671	5.32	CAEQFCR	100	71
					LPLRYPER		

					MESSISSSSEEMSLSK		
					NNVMLQW		
					QTDEIKDTR		
					LNEYNQLQLQAAHAQEQIR		
					LQNPSESSEPIPLESR		
					NESTQNCVVAEPEK		
					EEYMNGMNR		
P15291	B4GT1_HUMAN	Beta-1.4-galactosyltransferase 1	43.921	8.88	VAIIIPFR	99.9	17
					ETMLSDGLNSLTYQVLDVQR	100	
					GMSISRPNAVVGR	99.9	
					QQLDYGIYVINQAGDTIFNR	99.9	
					HISVAMDK	100	
P01034	CYTC_HUMAN	Cystatin-C	15.799	8.75	ALDFAVGEYNK	99.7	19
					LVGGPMDASVEEEGVRR		
P0DOY3	IGLC3_HUMAN	Immunoglobulin lambda constant 2	11.265	6.91	AAPSVTLFPPSSEELQANK	100	17
					AGVETTTPSK		
					SYSCQVTHEGSTVEK		
					YAASSYLSLTPEQWK		
P08571	CD14_HUMAN	Monocyte differentiation antigen CD14	40.076	5.84	FPAIQNLALR	100	35
					ELTLEDLK		
					ITGTMPPLPLEATGLALSSLR		
					VLSIAQAHSPAFSCEQVR		
					AFPALTSLDLSDNPGLGER		
					ATVNPSAPR		
					GLMAALCPHK		
					LKELTLEDLK		
					LTVGAAQVPAQLLVGALR		
					VLDLSCNR		
					VLAYSR		
P23280	CAH6_HUMAN	Carbonic anhydrase	35.366	6.41	IEEILDYLR	99.9	15
					LENSLLDHR	100	

					QSPINLQR	99.8	
					TTLTGLDVQDMLPR	99.8	
					YNPSLK	100	
P63261	ACTG_HUMAN	Actin. cytoplasmic 1	41.737	5.31	AGFAGDDAPR	100	15
					DSYVGDEAQSK		
					EITALAPSTMK		
					IIAPPER		
					SYELPDGQVITIGNER		
					QEYDESGPSIVHR		
					DLYANTVLSGGTTMYPGIADR		
					RGILTLK		
					AVFPSIVGRPR		
					GYSFTTTAER		
					VAPEEHPVLLTEAPLNPK		
					DLTDYLMK		
P11142	HSP7C_HUMAN	Heat shock cognate 71 kDa protein	68.807	5.37	DAGTIAGLNVLR	99.6	19
					VEIIANDQGNR	99.7	
					LLQDFFNGK	100	
					SFYPEEVSSMVLTK	100	
					NQVAMNPTNTVFDAK	100	
					NSLESYAFNMK	100	
					VQVEYKGETK	100	
					VCNPIITK	100	
					TTPSYVAFTDTER	99.7	
					IINEPTAAAIAYGLDKK	100	
P01009	A1AT_HUMAN	Alpha-1-antitrypsin	46.709	5.37	AVLTIDEK	100	35
					ITPNLAEFAFSLYR		
					LSITGTYDLK		
					LSSWVLLMK		
					SPLFMGK		
					VFSNGADLSGVTEEAPLK		

					FLEDVKK		
					QINDYVEK		
					SVLGQLGITK		
					LVDKFLEDVK		
					SASLHLPK		
					LGMFNIQHCK		
					TLNQPDSQLQLTTGNGLFLSEGLK		
Q6WN34	CRDL2_HUMAN	Chordin-like protein 2	47.493	8.32	ICPEDKADPGHSEISSTR	100	25
					VTASPDKVTK		
					AFGPLPCILCTCEDGR		
					VLVHTSVSPSPDNLR		
					VTCPTEYPCR		
					CTCSEGAHVSCYR		
					HPQDPCSSDAGR		
P06733	ENOA_HUMAN	Alpha-enolase	47.170	6.99	TIAPALVSK	99.8	28
					VNQIGSVTESLQACK	99.8	
					DATNVGDEGGFAPNILENK	100	
					EGLELLK	100	
					KLNVTEQEK	100	
					YISPDQLADLYK	98.6	
					AAVPSGASTGIYEALELR	100	
					GNPTVEVDLFTSK	100	
					IEEELGSK	100	
					IGAEVYHNLK	100	
P01780	HV307_HUMAN	Immunoglobulin heavy variable 3-7	12.942	8.65	NSLYLQMNSLR	99.8	32
					GRFTISR	100	
					GLEWVANIK	100	
					AEDTAVYYCAR	99.8	
P22897	MRC1_HUMAN	Macrophage mannose receptor 1	166.014	6.08	EGWNFYSNK	100	26
					FAWMDGSK		
					GEDLFFNYGNR		

					IFGFMEEER		
					IYGTTDNLCSR		
					LCLGVPSK		
					SCVSLNPGK		
					SSYSLMR		
					YFWTGLSDIQTK		
					DGYWADR		
					DYQYYFSK		
					GSGLWSR		
					IQMYFEWSDGTPVTFTK		
					SALTWHQAR		
					TWFESR		
					LGYICK		
					CPEDWGASSR		
					WYADCTSAGR		
					WENLECVQK		
					LFGYCPLK		
					FQWHEAETYCK		
					TNFWIGLFR		
					ALGGDLASINNKEEQQTIWR		
					LITASGSYHK		
					NFGDLVSIQSESEK		
					NWGQASLECLR		
					SQGPEIVEVEK		
					YTNWAADEPK		
					TGIAGGLWDVLK		
					YLNWLPGSPSAEPGK		
					GCEWPLGYICK		
					WVSESQIMSVAFK		
					TSLCFK		
					GYICQTR		
					FTHWNSDMPGR		

					GVHYTNWGK		
					MGSSLVSIESAAESSFLSYR		
P04114	APOB_HUMAN	Apolipoprotein B-100	515.614	6.58	AVSMPSFSILGSDVR	100	4
					TEVIPPLIENR		
					ATGVLYDYVNK		
					GVISIPR		
					LSLPDFK		
					SPAFTDLHLR		
					SVSDGIAALDLNAVANK		
					INPLALK		
					GFEPTLEALFGK		
					ATFQTPDFIVPLTDLR		
					AQIPILR		
					FIIPGLK		
					IEIPLPFGGK		
					DLKVEDIPLAR		
					TGISPLALIK		
					ITLPDFR		
					TLADLTLLDSPIK		
					SEYQADYESLR		
					ITENDIQIALDDAK		
P12273	PIP_HUMAN	Prolactin-inducible protein	16.573	5.40	TYLISSIPLQGAFNYK	99.9	37
					YTACLCDDNPK	99.9	
					ELGICPDDAAVIPIK	100	
					TVQIAAVVDVIR	100	
P07498	CASK_HUMAN	Kappa-casein	20.305	8.68	QYLPNSHPPTVVR	100	32
					RPAIAINNPYVPR		
					RPNLHPSFIAIPPK		
					TYYANPAVVRPHAQIPQR		
P06331	HV434_HUMAN		12.943	9.01	NQFSLK	99.7	45
					GRFTISR		

		Ig-like domain-containing protein (Fragment)			AEDTAVYYCAK		
					GLEWVAFIR		
					NTLYLQMNSLR		
					VTISVDTSK		
P01877	IGHA2_HUMAN	Immunoglobulin heavy constant alpha 2 (Fragment)	42.333	5.86	YLTWASR	99.8	66
					GETFSCMVGHEALPLAFTQK	100	
					SAVQGPPER	99.8	
					SGNTFRPEVHLLPPPSEELALNELVTLTCLAR	99.8	
					DASGATFTWTPSSGK	99.8	
					EKYLTWASR	99.8	
					GFSPKDVLVR	99.8	
					NFPPSQDASGDLYTTSSQLTLPATQCPDGK	99.8	
					QEPSQGTTTYAVTSILR	99.8	
					VAAEDWK	99.8	
					VPPPPPCCHPR	99.8	
					WLQGSQELPR	99.8	
P02750	A2GL_HUMAN	Leucine-rich alpha-2-glycoprotein	38.179	5.66	DLLLPQPDLR	100	31
					GQTLLAVAK		
					ALGHLDLSGNR		
					ENQLEVLEVSWLHGLK		
					GPLQLER		
					TLDLGENQLETLPPDLLR		
					VAAGAFQGLR		
					VLDLTR		
					DGFDISGNPWICDQNLSDLYR		
B9A064	IGLL5_HUMAN	Immunoglobulin lambda-like polypeptide 5	23.150	9.03	ANPTVTLFPPSSEELQANK	99.8	27
					VTVLGQPK		
					YAASSYLSLTPEQWK		
					SYSCQVTHEGSTVEK		

P01876	IGHA1_HUMAN	Immunoglobulin heavy constant alpha 1	42.848	6.08	YLTWASR	100	27
					GDTFSCMVGHEALPLAFTQK		
					SAVQGPPER		
					TFTCTAAYPESK		
					TPLTATLSK		
					SGNTFRPEVHLLPPPSEELALNELVTLTCLAR		
					DASGVTFTWTPSSGK		
					EKYLTWASR		
					GFSPKDVLVR		
					NFPPSQDASGDLYTTSSQLTLPATQCLAGK		
					QEPSQGTTTFAVTSILR		
					VAAEDWKK		
					WLQGSQELPR		
					DLCGCYSVSSVLPGCAEPWNHGK		
A0A075B6H7	KV37_HUMAN	Probable non-functional immunoglobulin kappa variable 3-7	12.784	5.14	LLIYGASTR	99.1	23
					EIVMTQSPPTLSLSPGER	99.8	
Q96DA0	ZG16B_HUMAN	Zymogen granule protein 16 homolog B	22.739	5.39	YFSTTEDYDHEITGLR	99.8	12
					VSVGLLLVK		
Q13410	BT1A1_HUMAN	Butyrophilin subfamily 1 member A1	58.961	5.32	EIPLSPMGEDSAPR	100	26
					FPSTSESR		
					KPLTICPIADGPER		
					KVEISIPASSLPR		
					VSPAVLVHR		
					ATLVQDGIAK		
					DGREQEAEQMPEYR		
					TPLPLAGPPR		
					VSDDGEYTCFFR		
					VTVIANAQDLSK		

					FDSWPCVLGR		
					TDWAIGVCR		
P01024	CO3_HUMAN	Complement C3	187.149	6.02	ACEPGVDYVYK DFDFVPPVVR FYYIYNEK GVFVLNKK GYTQQLAFR RIPIEDGSGEVVLSR VLLDGVQNPR SGSDEVQVGQQR SSLSVPYVIVPLK TGLQEVEVK TIYTPGSTVLYR VVPEGIR AGDFLEANYMNLQR DICEEQVNSLPGSITK LVLSSEK KQELSEAEQATR LMNIFLK SDDKVTLEER VFLDCCNYITELR VTIKPAPETEK DSCVGSLVVK EYVLPSFEVIVEPTEK IWDVVEK VHQYFNVELIQPGAVK VSHSEDDCLAFK VYAYYNLEESCTR LSINTHPSQKPLSITVR ILLQGTPVAQMTEDAVDAER IPIEDGSGEVVLSR	100	36

					ISLPESLK		
					KVLLDGVQNPR		
					LPYSVVR		
					SNLDEDIIAEENIVSR		
					TFISPIK		
					VELLHNPAFCSLATTK		
					VVLVAVDK		
					YYTYLIMNK		
					ASHLGLAR		
					AVLYNYR		
					AYYENSPQQVFSTEFEVK		
					LKGPLLNK		
					QPSSAFAAFVK		
					SGIPIVTSPYQIHFTK		
					TMQALPYSTVGNSNNYLHLSVLR		
					TVMVNIENPEGIPVK		
					VPVAVQGEDTVQSLTQGDGVAK		
					AELQCPQPAAR		
					FISLGEACK		
					APSTWLTAYVVK		
					EALKLEEK		
					LVAYYTLIGASGQR		
					QGALELIK		
					NTLIIYLDK		
A0A5H1ZRS9	A0A5H1ZRS9_HUMAN	Immunoglobulin kappa variable 2D-29	11.191	5.63	FSGSGSGTDFTLK	99.8	20
					FSGVPDR		
P15941	MUC1_HUMAN	Mucin-1 (Fragment)	25.565	6.96	NYGQLDIFPAR	97.9	20
					QGGFLGLSNIK	100	
					YVPPSSTDR	100	
					EGTINVHDVETQFNQYK	100	
					DISEMFLQIYK	100	

P04745	AMY1_HUMAN	Alpha-amylase 1	57.768	6.34	TGSGDIENYNDATQVR	97.6	15
					SSDYFGNGR	100	
					IYVSDDGK	100	
					ALVFVDNHDNQR	100	
					NVVDGQPFTNWYDNGSNQVAFGR	100	
					LSGLLDLALGK	100	
Q08431	MFGM_HUMAN	Lactadherin	43.104	8.47	EVTGIITQGAR	100	54
					NAVHVNLFETPVEAQYVR		
					NLFETPILAR		
					QITASSSYK		
					NPCHNGGLCEEISQEVR		
					VAYSNDSANWTEYQDPR		
					GDVFPSYTCTCLK		
					LASHEYLK		
					LYPTSCHTACTLR		
					MWVTGVVTQGASR		
					AGMVNAWTPSSNDDNPWIQVNLLR		
					FELLGCELNGCANPLGLK		
					ILPVAWHNR		
					VTFLGLQHWVPELAR		
					NFGSVQFVASYK		
P00709	LALBA_HUMAN	Alpha-lactalbumin	16.225	4.70	CELSQLLK	100	49
					FLDDDITDDIMCAK	100	
					GIDYWLAHK	100	
					KILDIK	100	
					LEQWLCEKL	100	
					ALCTEKLEQWLCEK	100	
					NICDISCDK	99.1	
P25311	ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	34.258	5.58	AYLEEECPATLR	99.8	23
					EIPAWVPFDPAAQITK	100	
					CLAYDFYPGK	100	

					YSLTYIYTGLSK	100	
					AGEVQEPELR	100	
					SSGAFWK	100	
A0A0G2JM16	A0A0G2JM16_HUMAN	Mucin-4	728.519	4.94	IGLASALQPR	98.1	2
					SLEPFTLEILAR	100	
					GYDLVYSPQSGFTCVSPCSR	100	
					FQPVSIGR	100	
					TVDFTSPLFKPATGFPLGSSLR	100	
					LGTLDMR	100	
					SSSLGPVTVQWLLEPHDAIR	100	
					LECLQWLK	99.4	
P61626	LYSC_HUMAN	Lysozyme C	16.536	9.28	TPGAVNACHLSCSALLQDNIADAVACAK	100	54
					WESGYNTR		
					GISLANWMCLAK		
					LGMDGYR		
					STDYGIFQINSR		
					AWVAWR		
					YWCNDGK		
P07602	SAP_HUMAN	Prosaposin	58.440	5.06	GCSFLPDPYQK	98.8	13
					LVGYLDR	99.8	
					NVIPALELVEPIKK	100	
					SDVYCEVCEFLVK	100	
					LPALTVHVTQPK	100	
					LGPGMADICK	100	
P49327	FAS_HUMAN	Fatty acid synthase	273.198	6.01	AGLYGLPR	100	29
					AQVADVVVSR		
					ADEASELACPTPK		
					AINCATSGVVGLVNCLR		
					EDGLAQQQTQLNLR		
					GLVQALQTK		
					GVDLVLNSLAEEK		

					LLEQGLR		
					MVVPGLDGAQIPR		
					FCFTPHTEEGCLSER		
					HGLYLPTR		
					SFYGSTLFLCR		
					TGTVSLEVR		
					VGDPQELNGITR		
					VVVQVLAEEPEAVLK		
					LPEDPLLSGLLDSPALK		
					LSPDAIPGK		
					ACLDTAVENMPSLK		
					AFDTAGNGYCR		
					EQGVTFPSGDIQEQLIR		
					VLQGDLVMNVYR		
					VYQWDDPDPR		
					YSGTLNLDR		
					LYTLQDK		
					WTSQDSLLGMEFSGR		
					GILADEDSSRPVWLK		
					TGGAYGEDLGADYNLSQVCDGK		
					LQVVDQPLPVR		
					QEPLLIGSTK		
					GTPLISPLIK		
					LSIPTYGLQCTR		
					VLEALLPLKGLEER		
					FDASFFGVHPK		
					FPQLDSTSFANSR		
					GNAGQSNYGFANSAMER		
					SEGVVAVLLTKK		
					SDEAVKPFGLK		
					SLLVNPEGPTLMR		
					AALQEELQLCK		

					DGAWGAFR		
					DGLLENQTPEFFQDVCKPK		
					DNLEFFLAGIGR		
					DPSQQELPR		
					EGGFLLLHTLLR		
					GYAVLGGER		
					LGMLSPEGTCK		
					LHLSGIDANPNALFPPVEFPAPR		
					LLSAACR		
					QELSFAAR		
					RPTPQDSPIFLPVDDTSFR		
					RQQEQQVPILEK		
					FLEIGK		
					QVQPEGPYR		
					VFTTVGSAEKR		
					VTAIHIDPATHR		
					DGVVRPLK		
					GDLSSIR		
					ELNLVLSVR		
					FRDSILR		
					GMGLSLMR		
					IPGLLSPHPLLQLSYTATDR		
					VMGLVPAK		
P01857	IGHG1_HUMAN	Immunoglobulin heavy constant gamma 1 (Fragment)	43.911	8.46	ALPAPIEK	100	29
					DTLMISR		
					GPSVFPLAPSSK		
					NQVSLTCLVK		
					STSGGTAALGCLVK		
					FNWYVDGVEVHNAK		
					TTPPVLDSDGSFFLYSK		
					GFYPSDIAVEWESNGQPENNYK		

					TPEVTCVVVDVSHEDPEVK		
					EPQVYTLPPSRDELTK		
					VVSVLTVLHQDWLNGK		
P16671	CD36_HUMAN	Platelet glycoprotein 4	52.063	8.21	SQVLQFFSSDICR	99.9	14
					AFASPVENPDNYCFCTEK	100	
					GIPVYR	100	
					SSMFQVR	100	
					VAIIDTYK	99.8	
					LQVNLLVKPSEK	99.8	
P00450	CERU_HUMAN	Ceruloplasmin	108.823	5.41	DIASGLIGPLIICK	99.8	9
					GAYPLSIEPIGVR	99.8	
					DLYSGLIGPLIVCR	100	
					EVGPTNADPVCLAK	100	
					NNEGTYYSPNYNPQSR	100	
					VNKDDEEFIESNK	100	
					EYTDASFTNRK	100	
P24821	TENA_HUMAN	Tenascin	240.850	4.79	AAIDSYR	100	25
					ETFTTGLDAPR		
					FTTDLDSPR		
					GAFWYR		
					IQALNGPLR		
					ITAQGQYELR		
					KQSEPLEITLLAPER		
					LLDPQEFTLSGTQR		
					TTLTGLRPGTEYGIGVSAVK		
					YAPISGGDHAEVDVPK		
					AYAAGFGDR		
					ESNPATINAATELDTPK		
					FSVGDAK		
					LIPGVEYLVSIIAMK		
					SIPVSAR		

					ASTEQAPELENLTVTEVGWDGLR		
					LPVGSQCSVDLESASGEK		
					AATHYTITIR		
					AATPYTVSIYGVIQGYR		
					VEGYSGTAGDSMAYHNGR		
					ACGCAAAPDVK		
					APTAQVESFR		
					AVDIPGLEAATPYR		
					AVDIPGLK		
					EQCTAGAGCCLQPATGR		
					GLEPGQEYNVLLTAEK		
					ITYVPITGGTPSMVTVDGTK		
					LDAPSQIEVK		
					LEELENLVSSLR		
					SMEIPGLR		
					RSQTVSAIATTAMGSPK		
					STDLPGLK		
					VATYLPAPEGLK		
					VFAILENK		
					VSIYGVIR		
					ENFYQNWK		
					IFAEKGPQK		
					TPVLSAEASTAK		
					TVSGNTVEYALTDLEPATEYTLR		
					VSQTDNSITLEWR		
					AGTPYTVTLHGEVR		
					DHGETAFAVYDK		
					CECDDGFTGADCGELK		
Q08380	LG3BP_HUMAN	Galectin-3-binding protein	65.332	5.07	YSSDYFQAPSDYR	100	27
					AVDTWSWGER		
					STSSFPCPAGHFNGFR		

					TLQALEFHTVPFQLLAR		
					LASAYGAR		
					ASHEEVEGLVEK		
					ELSEALGQIFDSQR		
					SDLAVPSELALLK		
					STHTLDLSR		
					VEIFYR		
					RIDITLSSVK		
					KSQLVYQSR		
					IYTSPTWSAFVTDSSWSAR		
A0A0B4J2B5	A0A0B4J2B5_HUMAN	Immunoglobulin heavy variable 3/OR16-9	10.656	8.66	EVQLVESGGGLVQPGGSLR	99.8	49
					NSLYLQMNSLR		
					GRFTISR		
					AEDTAVYYCVK		
P01591	IGJ_HUMAN	Immunoglobulin J chain	18.098	4.59	CYTAVVPLVYGGETK	100	42
					MVETALTPDACYPD		
					FVYHLSDLCK		
					IIVPLNNR		
					IVLVDNK		
					SSEDPNEDIVER		
P10909	CLUS_HUMAN	Clusterin	52.495	5.88	ALQEYR	100	32
					FMETVAEK		
					TLLSNLEEAK		
					LFDSDPITVTVPVEVSR		
					EIQNAVNGVK		
					TLLSNLEEAKK		
					ASSIIDELFQDR		
					EILSVDCSTNNPSQAK		
					ELDESLQVAER		
					KTLLSNLEEAK		
					KYNELLK		

					EPQDTYHYLPFSLPHR		
					IDSLLENDR		
P05814	CASB_HUMAN	Beta-casein	25.381	5.33	GRVMPVLK	100	86
					SPTIPFFDPQIPK		
					VLPIPQQVVPYPQR		
					IYPSFQPQPLIYPFVEPIPYGFLPQNILPLAQPAVVLP VPQPEIMEVPK		
					LTDLENLHLPLPLLQPLMQQVPQPIPQTLALPPQPL WSVPQPK		
					AKDTVYTK		
					VMPVLK		
					AVPVQALLLNQELLLNPTHQIYPVTQPLAPVHNPISV		
					ETIESLSSSEESITEYK		
P47989	XDH_HUMAN	Xanthine dehydrogenase/oxidase	146.426	7.86	DPPADVQLFQEVPK	100	29
					EGDLTHFNQK		
					ELLDLK		
					IPAFGSIPIEFR		
					LDPTFASATLLFQK		
					ELFRLDSPATPEK		
					LEGFTLPR		
					LGCGEGGCGACTVMLSK		
					LGQENLEDK		
					LVFFVNGR		
					LVVGNTEIGIEMK		
					NADPETTLLAYLR		
					NNSFYGPELK		
					TLVDAVAK		
					TNLPSNTAFR		
					FYLTVLQK		
					ITYEELPAIITIEDAIK		

					MLGVPANR		
					SVASVGGNIITASPISDLNPVFMASGAK		
					MVQVASR		
					GEAGEMELFVSTQNTMK		
					TADKLVFFVNGR		
					CMLDRDEDMLITGGR		
					EGEYFSAFK		
					GVLEQLR		
					LDSPATPEK		
					STVVSTAVALAAYK		
					TVQMDHTFFPGYR		
					VTWIQASTLK		
					YENELSLR		
					ALKIPTSK		
					HPFLAR		
					NQPEPTMEEIENAFQGNLCR		
					GLCIIPTK		
					KLGLSGTK		
					ALFHMDNCYK		
P00738	HPT_HUMAN	Haptoglobin	45.204	6.13	VTSIQDWVQK	100	32
					YVMLPVADQDQCIR		
					DIAPTLTLYVGKK		
					AVGDKLPECEAVCGKPK		
					KQLVEIEK		
					SPVGVQPILNEHTFCAGMSK		
					VMPICLPSKDYAEVGR		
					VGYVSGWGR		
					TEGDGVYTLNNEK		
					ILGGHLDAK		
P01834	IGKC_HUMAN	Immunoglobulin kappa constant	11.764	6.11	RTVAAPSVFIFPPSDEQLK	100	80
					SGTASVVCLLNNFYPR		

					VDNALQSGNSQESVTEQDSK		
					VYACEVTHQGLSSPVTK		
					DSTYSLSSTLTLSK		
					TVAAPSVFIFPPSDEQLK		
P23284	PPIB_HUMAN	Peptidyl-prolyl cis-trans isomerase B	23.743	9.25	TVDNFVALATGEK	97.8	26
					VLEGMEVVR	100	
					DTNGSQFFITTVK	100	
					VYFDLR	100	
					IGDEDVGR	100	
					VIFGLFGK	98.3	
O00391	QSOX1_HUMAN	Sulfhydryl oxidase 1	82.578	9.05	NGSGAVFPVAGADVQTLR	100	19
					EVLPAIR		
					IYMADLESALHYILR		
					IPYSFFK		
					FVAVLAK		
					DFNIPGFPTVR		
					EVALDLSQHK		
					IEVGRFPVLEGQR		
					LAGAPSEDPQFPK		
					SALYSPSDPLTLLQADTVR		
					VLNTEANVVR		
					SFYTAYLQR		
P01619	KV320_HUMAN	Immunoglobulin kappa variable 3-20	12.557	4.85	ATGIPDR	99.8	28
					LLIYGASSR	99.8	
					FSGSGSGTDFTLTISR	100	
P0C0L5	CO4B_HUMAN	Complement C4-B	187.671	6.89	AEFQDALEK	100	38
					ASSFLGEK		
					DHAVDLIQK		
					GLCVATPVQLR		
					ITQVLHFTK		
					KYVLPNFEVK		

					LGQYASPTAK		
					LNMGITDLQGLR		
					LTSLSDR		
					VLSLAQEQVGGSPEK		
					SCGLHQLLR		
					VDFTLSSER		
					LELSVDGAK		
					LVNGQSHISLSK		
					FACYYPR		
					SHALQLNNR		
					VLQIEK		
					AEMADQAAAWLTR		
					ASAGLLGAHAAAITAYALTLTK		
					LQETSNWLLSQQQADGSFQDLSPVIHR		
					EMSGSPASGIPVK		
					GLQDEDGYR		
					GQIVFMNR		
					GSFEFPVGDAVSK		
					LLATLCSAEVCQCAEGK		
					QGSFQGGFR		
					TTNIQGINLLFSSR		
					VFALDQK		
					VGDTLNLNLR		
					EPFLSCCQFAESLR		
					FGLLDEDGKK		
					HLVPGAPFLLQALVR		
					KADGSYAAWLSR		
					VEYGFQVK		
					CSVFYGAPSK		
					GPEVQLVAHSPWLK		
					GSSTWLTAFVLK		
					VDVQAGACEGK		

					YIYGKPVQGVAYVR		
					GHLFLQTDQPIYNPGQR		
					LLLFSPSVVHLGVPLSVGVQLQDVPR		
					ALEILQEEDLIDEDDIPVR		
					LPMSVR		
					AACAQLNDFLQEYGTQGCQV		
					DFALLSLQVPLKDAK		
					EELVYELNPLDHR		
					SFFPENWLWR		
Q13228	SBP1_HUMAN	Methanethiol oxidase	52.392	5.94	LVLPSLISSR	99.8	15
					DGLIPLEIR	100	
					SPQYCQVIHR	100	
					IYVVDVGSEPR	99.8	
					EEIVYLPCIYR	100	
					NTGTEAPDYLATVDVDPK	100	
P02768	ALBU_HUMAN	Serum albumin	69.366	5.67	AAFTECCQAADK	100	66
					AEFAEVSK		
					AVMDDFAAFVEK		
					CCTESLVNR		
					DLGEENFK		
					FQNALLVR		
					KVPQVSTPTLVEVSR		
					LDELRDEGK		
					LVNEVTEFAK		
					QNCELFEQLGEYK		
					QTALVELVK		
					TCVADESAENCDK		
					TYETTLEK		
					YICENQDSISSK		
					AACLLPK		
					VFDEFKPLVEEPQNLIK		

					LVTDLTK		
					ADDKETCFAEEGK		
					CCAAADPHECYAK		
					DDNPNLPR		
					KYLYEIAR		
					LCTVATLR		
					LKECCEKPLLEK		
					SLHTLFGDK		
					DVFLGMFLYEYAR		
					SHCIAEVENDEMPADLPSLAADFVESK		
					TPVSDRVTK		
					LVAASQAALGL		
					ETYGEMADCCAK		
					FKDLGEENFK		
					NECFLQHKDDNPNLPR		
					HPYFYAPELLFFAK		
					LSQRFPK		
					RPCFSALEVDETYVPK		
					DAHKSEVAHR		
					RHPDYSVVLLLR		
P11021	BIP_HUMAN	Endoplasmic reticulum chaperone BiP	72.334	5.01	SQIFSTASDNQPTVTIK	100	20
					ELEEIVQPIISK	100	
					NQLTSNPENTVFDAK	100	
					VTHAVVTVPAYFNDAQR	100	
					ITPSYVAFTPEGER	97.6	
					IINEPTAAAIAYGLDKR	100	
					VEIIANDQGNR	100	
					TWNDPSVQQDIK	100	
					LTPEEIER	100	
					NELESYAYSLK	100	
			11.167	8.05	EVQLVESGGGLVQPGGSLR	98.7	44

A0A4W8ZXM2	A0A4W8ZXM2_HUMAN	Immunoglobulin heavy variable 3-72			GLEWVGR	98.7	
					GRFTISR	99.8	
					TEDTAVYYCAR	99.8	
Q14697	GANAB_HUMAN	Neutral alpha-glucosidase AB	106.875	5.74	LVAIVDPHIK	99.9	3
					LDLLEDR	98.7	
					LSFQHDPETSVLVLR	99.7	
P02763	A1AG1_HUMAN	Alpha-1-acid glycoprotein 1	23.512	5.00	EQLGEFYEALDCLR	99.9	16
					SDVVYTDWKK	98.9	
					TEDTIFLR	99.7	
P04004	VTNC_HUMAN	Vitronectin	54.306	5.55	GQYCYELDEK	98.6	11
					DVWGIEGPIDAAFTR	99.8	
					SIAQYWLGCPAPGHL	99.7	
					IYISGMAPRPSLAK	100	
P02748	CO9_HUMAN	Complement component C9	63.174	5.43	LSPIYNLVPVK	98.8	6
					AIEDYINEFSVR	99.2	
					VVEESELAR	99.9	
P04217	A1BG_HUMAN	Alpha-1B-glycoprotein	54.253	5.63	LLELTGPK	99.6	12
					ELLVPR	99.9	
					VTLTCVAPLSGVDFQLR	100	
					ATWSGAVLAGR	99.9	
					GVTFLLR	100	
					SGLSTGWTQLSK	100	
P22079	PERL_HUMAN	Lactoperoxidase	73.959	8.22	AGFVCPTPPYK	100	4
					VGPLLACLLGK	99.1	
					NGFPLPLAR	99.9	
P04003	C4BPA_HUMAN	C4b-binding protein alpha chain	67.033	6.24	LSLEIEQLELQR	98.9	8
					YTCLPGYVR	99.8	
					EDVYVVGTVLR	100	
					FSAICQGDGTWSPR	99.8	
Q16851	UGPA_HUMAN		57.807	8.15	SFENSLGINVPR	100	30

		UTP--glucose-1-phosphate uridylyltransferase			GGTLTQYEGK	100	
					AMSQDGASQFQEVIR	100	
					IQRPPEDSIQPYEK	100	
					LGSSFTK	100	
					VQDYLR	100	
					ESLLPVAK	100	
					GPSVDWGK	100	
					GTVIIIANHGDR	100	
					LVEIAQVPK	99	
					GLPDNISSVLNK	100	
					EFPTVPLVK	100	
					NENTFLDLTVQQIEHLNK	100	
					INKESLLPVAK	100	
O00300	TR11B_HUMAN	Tumor necrosis factor receptor superfamily member 11B	46.026	8.71	IIQDIDLCENSVQR	99.4	13
					FTPNWLSVLVDNLPGTK	100	
					LLSLWR	100	
					SCPPGFGVVQAGTPER	100	
P02774	VTDB_HUMAN	Vitamin D-binding protein	53.021	5.16	VLEPTLK	99.2	18
					SLGECCDVEDSTTCFNAK	100	
					HLSLLTTLSNR	100	
					EVVSLTEACCAEGADPDCYDTR	100	
					LCDNLSTK	100	
					VCSQYAAYGEK	99.9	
					KLCMAALK	100	
O75888	TNF13_HUMAN	Protein TNFSF12-TNFSF13	36.588	9.79	GLQAQGYGVR	98.4	37
					QETLFR	99.8	
P05164	PERM_HUMAN	Myeloperoxidase	83.871	9.19	NNIFMSNSYPR	100	10
					NQADCIPFFR	100	
					IPCFLAGDTR	99.6	
					IANVFTNAFR	100	
					QALAQISLPR	100	

					VGPLLACIIGTQFR	100	
					VVLEGGIDPILR	100	
O15232	MATN3_HUMAN	Matrilin-3	52.816	5.99	IIDTLDIGPADTR	99.8	9
					VAVVNYASTVK	99	
					RPSPAAPDGAPASGTSEPGR	99.8	
P62987	RL40_HUMAN	Polyubiquitin-B	19.016	6.56	MQIFVK	99.8	10
					TITLEVEPSDTIENVK	98	
					ESTLHLVLR	99.8	
O00299	CLIC1_HUMAN	Chloride intracellular channel protein 1	26.923	5.09	LAALNPESNTAGLDIFAK	98.5	11
					GFTIPEAFR	98.4	
P62937	PPIA_HUMAN	Peptidyl-prolyl cis-trans isomerase	13.022	7.68	IIPGFMCQGGDFTR	99.8	12
					VSFELFADK		
P01019	ANGT_HUMAN	Angiotensinogen	53.154	5.87	DPTFIPAPIQAK	99.4	8
					ALQDQLVLVAAK	97.5	
					QPFVQGLALYTPVVLPR	99.9	
P02749	APOH_HUMAN	Beta-2-glycoprotein 1	38.298	8.37	ATVVYQGER	98.6	12
					VCPFAGILENGAVR	99.8	
					TCPKPDDLPFSTVVPLK	100	
P01033	TIMP1_HUMAN	Metalloproteinase inhibitor 1	16.057	8.47	GFQALGDAADIR	99.8	23
					LQDGLLHITTCSFVAPWNSLSLAQR	99.8	
					EPGLCTWQSLR	97.8	
Q96S86	HPLN3_HUMAN	Hyaluronan and proteoglycan link protein 3	47.756	6.08	LTLTEAR	99.6	8
					VGQLFAAWK	99.8	
					EACQEDDATIAK	100	
P02649	APOE_HUMAN	Apolipoprotein E	36.153	5.52	SELEEQLTPVAEETR	100	17
					AKLEEQAQQIR	100	
					LGPLVEQGR	99.9	
					LAVYQAGAR	99.9	
					LQAEAFQAR	99.9	
P68104	EF1A1_HUMAN		50.141	9.10	IGGIGTVPVGR	97.5	18

		Elongation factor 1-alpha 1			YYVTIIDAPGHR	100	
					STTTGHLIYK	100	
					QLIVGVNK	100	
					LPLQDVYK	100	
					QTVAVGVIK	100	
					VETGVLKPGMVVTFAPVNVTTEVK	100	
P02790	HEMO_HUMAN	Hemopexin	51.676	6.43	NFPSPVDAAFR	99.8	14
					GGYTLVSGYPK	100	
					LLQDEFPGIPSPLDAAVECHR	100	
					VDGALCMEK	100	
					YYCFQGNQFLR	99.8	
P13987	CD59_HUMAN	CD59 glycoprotein (Fragment)	13.343	5.18	AGLQVYNK	99.4	7
					FEHCNFNDVTTR	98.1	
					LRENELTYYCCK	100	
P01133	EGF_HUMAN	Pro-epidermal growth factor	112.704	5.53	ITAVSLDVLDKR	99.6	3
					YPANVAVDPVER	99.8	
					LFWTDTGINPR	99.8	
P05156	CFAI_HUMAN	Complement factor I	65.059	7.72	AQLGDLPWQVAIK	99.8	19
					GLETSLAECTFTK	100	
					VFSLQWGEVK	99.8	
P18065	IBP2_HUMAN	Insulin-like growth factor-binding protein 2	34.813	6.89	LEGEACGVYTPR	99.8	8
					TPCQQELDQVLER	98.6	
P05413	FABPH_HUMAN	Fatty acid-binding protein. heart	14.787	6.34	LGVEFDETTADDR	100	74
					NGDILTLK		
					NTEISFK		
					SIVTLDGGK		
					SLGVGFATR		
					WDGQETTLVR		
					NFDDYMK		
					LILTLTHGTAVCTR		

					LVHLQK		
					QVASMTKPTTIIEK		
P08238	HS90B_HUMAN	Heat shock protein HSP 90-beta	83.267	4.96	ADLINNLGTIAK	99	16
					EQVANSAFVER	100	
					SIYYITGESK	100	
					SLTNDWEDHLAVK	100	
					IDIIPNPQER	100	
					ALLFIPR	100	
					ELISNASDALDKIR	100	
					NPDDITQEEYGEFYK	100	
					GVVDSEDLPLNISR	100	
					TLTLVDTGIGMTK	100	
Q9H4G4	GAPR1_HUMAN	Golgi-associated plant pathogenesis-related protein 1	14.212	9.44	ASASDGSSFVVAR	99.8	17
					EAQQYSEALASTR		
Q9H173	SIL1_HUMAN	Nucleotide exchange factor SIL1	52.085	5.22	LGGLQVLR	100	10
					VLQTLGVLLTTCR		
					LLVILATEQPLTAK		
					VVTLLYDLVTEK		
P00747	PLMN_HUMAN	Plasminogen	90.567	7.04	LSSPAVITDK	99.8	10
					EAQLPVIENK	99.8	
					WELCDIPR	99.8	
					NPDGDVGGPWCYTTNPR	99.9	
P07195	LDHB_HUMAN	L-lactate dehydrogenase B chain	37.405	5.72	LIAPVAEEEATVPNNK	99.8	23
					FIIPQIVK	100	
					SADTLWDIQK	100	
					IVADKDYSVTANSK	100	
					IVVVTAGVR	100	
					VIGSGCNLDSAR	99.4	
					GLTSVINQK	100	
P15311	EZRI_HUMAN	Ezrin	69.373	5.94	APDFVFYAPR	99.8	6

					IGFPWSEIR	99	
					IALLEEAR	100	
					ALQLEEER	100	
Q96KP4	CNDP2_HUMAN	Cytosolic non-specific dipeptidase	52.879	5.66	TVFGVEPDLTR	99.3	13
					TGQEIPVNVR	99.5	
					QLGGSVELVDIGK	100	
					QKLPDGSEIPLPPILLGR	100	
					YNYIEGTK	99.8	
Q6X4U4	SOSD1_HUMAN	Sclerostin domain-containing protein 1	23.306	9.93	ITVVTACK	99.8	9
					IQLQCQDGSTR	99.6	
P00751	CFAB_HUMAN	cDNA FLJ55673. highly similar to Complement factor B	140.943	6.67	DISEVVTPR	99.9	66
					ISVIRPSK	99.8	
					KCLVNLIEK	100	
					VASYGVKPR	100	
					VSEADSSNADWVTK	100	
					YGQTIRPICLPCTEGTTR	100	
					LEDSVTYHCSR	99.9	
					YGLVTYATYPK	99.2	
					FLCTGGVSPYADPNTCR	100	
					LLQEGQALEYVCPSGFYPYPVQTR	100	
					STGSWSTLK	100	
					DAQYAPGYDK	100	
P13639	EF2_HUMAN	Elongation factor 2	95.340	6.42	FSVSPVVR	100	6
					YEWDVAEAR	100	
					VNFTVDQIR	99.9	
					TFCQLILDPIFK	98.4	
					VFSGLVSTGLK	99.9	
P02647	APOA1_HUMAN	Apolipoprotein A-I	30.778	5.56	LLDNWDSVTSTFSK	100	32
					AKPALEDLR		
					DSGRDYVSQFEGSALGK		
					VQPYLDDFQK		

					VSFLSALEEYTK		
					QGLLPVLESFK		
					THLAPYSDELR		
P04075	ALDOA_HUMAN	Fructose-bisphosphate aldolase A	39.818	8.93	GILAADESTGSIAK	99.8	20
					VLAAVYK	100	
					AAQEEYVKR	100	
					GVVPLAGTNGETTTQGLDGLSER	100	
					ELSDIAHR	100	
					ALANSLACQGK	100	
P60174	TPIS_HUMAN	Triosephosphate isomerase	30.79	5.65	IAVAAQNCYK	100	29
					QSLGELIGTLNAAK	100	
					SNVSDAVAQSTR	99.8	
					VVLAYEPVWAIGTGK	99.8	
					VPADTEVVCAPPTAYIDFAR	99.8	
					IIYGGSVTGATCK	100	
P06396	GELS_HUMAN	Gelsolin	84.745	5.72	AGALNSNDAFVLK	100	6
					TGAQELLR		
					QTQVSVLPEGGETPLFK		
					HVVPNEVVVQR		
P27105	STOM_HUMAN	Erythrocyte band 7 integral membrane protein	31.732	7.71	LPVQLQR	99.8	5
					LLAQTTLR		
P07900	HS90A_HUMAN	Heat shock protein HSP 90-alpha	84.663	4.94	ADLINNLGTIAK	99.2	12
					SLTNDWEDHLAVK	100	
					ALLFVPR	99.2	
					ELISNSSDALDKIR	99.9	
					GVVDSEDLPLNISR	100	
					NPDDITNEEYGEFYK	100	
					TLTIVDTGIGMTK	100	
P02751	FINC_HUMAN	Fibronectin	272.312	5.31	IYLYTLNDNAR	100	3

					GEWTCIAYSQLR		
					STTPDITGYR		
					LTVGLTR		
					VPGTSTSATLTGLTR		
					NTFAEVTGLSPGVTYYFK		
P01860	IGHG3_HUMAN	Immunoglobulin heavy constant gamma 3 (Fragment)	41.325	8.23	ALPAPIEK	100	29
					DTLMISR		
					GPSVFPLAPCSR		
					NQVSLTCLVK		
					SCDTPPPCPR		
					TPLGDTTHTCPR		
					STSGGTAALGCLVK		
					VVSVLTVLHQDWLNGK		
P59665	DEF1_HUMAN	Neutrophil defensin 1	10.201	6.54	IPACIAGER	99.9	19
					YGTCIYQGR		
P01023	A2MG_HUMAN	Alpha-2-macroglobulin	163.289	5.98	VTAAPQSVCALR	100	2
					TEHPFTVEEFVLPK		
P15531	NDKA_HUMAN	Nucleoside diphosphate kinase A (Fragment)	9.371	5.81	TFIAIKPDGVQR	98.5	13
					GLVGEIIK	99.8	
P80188	NGAL_HUMAN	Neutrophil gelatinase-associated lipocalin	22.788	9.02	SYPGLTSYLVR	99.8	13
					VPLQQNFQDNQFQGK		
P14618	KPYM_HUMAN	Pyruvate kinase	49.898	7.95	GDLGIEIPAEK	100	7
					LDIDSPPITAR	98.1	
					NTGIICTIGPASR	100	
P30086	PEBP1_HUMAN	Phosphatidylethanolamine-binding protein 1	21.056	7.01	GNDISSGTVLSDYVGSGPPK	97.8	23
					LYEQLSGK	100	
					LYTLVLTDPDAPSRK	99.7	
P53396	ACLY_HUMAN	ATP-citrate synthase	120.842	6.95	EILIPVFK	99.8	7
					FICTTSAIQNR	99.8	
					VDATADYICK	100	

					EAGVFVPR	100	
					FGGALDAAAK	100	
					SGGMSNELNNIISR	100	
					TIAIIAEGIPEALTR	100	
Q71U36	TBA1A_HUMAN	Tubulin alpha chain	57.730	4.94	DVNAAIATIK	99.5	7
					EIIDLVLDR	100	
					AVFVDLEPTVIDEVR	99.9	
P36871	PGM1_HUMAN	Phosphoglucomutase -1	61.451	6.30	EAIQLIAR	100	10
					IALYETPTGWK	99.8	
					VDLGVLGK	99.8	
					ADNFEYSDPVDGSISR	100	
					TIEEYAVCPDLK	100	
P25774	CATS_HUMAN	Cathepsin S	37.495	7.64	GIDSDASYPYK	99.8	11
					LVSLSAQNLVDCSTEK	99.8	
					GPVSVGVDAR	100	
P29401	TKT_HUMAN	Transketolase	49.911	7.58	ILATPPQEDAPSVDIANIR	99.9	10
					LDNLVAILDINR	100	
					VLDPFTIKPLDR	99.9	
					IIALDGDTK	100	
					KLILDSAR	100	
Q06830	PRDX1_HUMAN	Peroxiredoxin-1 (Fragment)	18.976	8.27	ADEGISFR	100	10
					QITVNDLPVGR	97.6	
					GLFIIDDKGILR	100	
					TIAQDYGVLK	100	
P15328	FOLR1_HUMAN	Folate receptor alpha	29.818	8.32	VLNVPLCK	97.5	7
					TELLNVCMNAK	99.8	
P30101	PDIA3_HUMAN	Protein disulfide-isomerase A3	56.784	5.61	YGVSGYPTLK	99.8	6
					LAPEYEAAATR		
					ELSDFISYLQR		
P00558	PGK1_HUMAN		44.615	8.30	LGDVYVNDAFGTAHR	99.7	18

		Phosphoglycerate kinase 1			LTLDKLDVK	99.7	
					ACANPAAGSVILLENLR	100	
					VLNNMEIGTSLFDEEGAK	100	
					ALESPERPFLAILGGAK	100	
P05546	HEP2_HUMAN	Heparin cofactor 2	57.072	6.26	TLEAQLTPR	99.8	3
					IAIDLFK	98.2	
P14625	ENPL_HUMAN	Endoplasmin	92.471	4.73	SILFVPTSAPR	98.6	6
					GVVDSDDLPLNVSR	100	
					LIINSLYK	98.7	
					ELISNASDALDKIR	98.6	
Q14195	DPYL3_HUMAN	Dihydropyrimidinase-related protein 3	61.964	6.04	GMTTVDDFFQGTK	100	17
					SAADLISQAR		
					IFNLYPR		
					MDENQFVAVTSTNAAK		
					GAPLVVICQGK		
					GMYDGPVFDLTTTPK		
					MSVIWDK		
					AITIASQTNCPLYVTK		
Q6UXI9	NPNT_HUMAN	Nephronectin (Fragment)	66.915	8.78	GDVFIPR	99.7	8
					LVLPLGR	98.4	
P40925	MDHC_HUMAN	Malate dehydrogenase. cytoplasmic	23.038	6.89	GEFVTTVQQR	99.8	12
					FVEGLPINDFSR	100	
					VIVVGNPANTNCLTASK	99.8	
P20061	TCO1_HUMAN	Transcobalamin-1	48.207	4.85	TFLDINK	100	10
					GTSAVNVVLSLK	99.6	
					NGENLEVR	100	
					LKPLLNTMIQSNYNR	100	
P21399	ACOC_HUMAN	Cytoplasmic aconitate hydratase	98.401	6.23	NIEVPFKPAR	99.8	4
					YQQAGLPLIVLAGK	98.9	

					VLLEAAIR	99.8	
P43490	NAMPT_HUMAN	Nicotinamide phosphoribosyltransferase	55.522	6.69	LLPPYLR	100	10
					VLEILGK	100	
					YLLETSGNLDGLEYK	100	
					GTDTVAGLALIK	99.7	
P63104	1433Z_HUMAN	14-3-3 protein zeta/delta	15.697	4.73	FLIPNASQAESK	99.8	14
					SVTEQGAELSNEER	99.7	
					NLLSVAYK	99.8	
P62820	RAB1A_HUMAN	Ras-related protein Rab-1A	22.172	5.93	EFADSLGIPFLETSAK	99.8	19
					LLLIGDSGVGK	99.7	
					LQIWDTAGQER	100	
P23396	RS3_HUMAN	40S ribosomal protein S3	26.688	9.68	AELNEFLTR	99.7	12
					GLCAIAQAESLR	99.8	
					TEIIILATR	99.8	
P07237	PDIA1_HUMAN	Protein disulfide-isomerase	56.784	4.69	ENLLDFIK	100	10
					VDATEESDLAQQYGVR		
					SNFAEALAAHK		
					ILEFFGLK		
					TVIDYNGER		
P06727	APOA4_HUMAN	Apolipoprotein A-IV	45.399	5.18	ISASAEELR	99.5	5
					LEPYADQLR	99.9	

a. theorical value

Supplementary Table 3. Differentially abundant proteins in human milk, in colostrum and mature groups, where “up” corresponds to “upregulated protein”

UniProt	ID	Protein name	MW (kDa)[a]	pI[a]	Colostrum	Mature
P63104	1433Z_HUMAN	14-3-3 protein zeta/delta	15.697	4.73		up
P23396	RS3_HUMAN	40S ribosomal protein S3	26.688	9.68		up
P63261	ACTG_HUMAN	Actin, cytoplasmic 1	41.737	5.31		up
P01011	AACT_HUMAN	Alpha-1-antichymotrypsin	47.653	5.32		up
P04217	A1BG_HUMAN	Alpha-1B-glycoprotein	54.253	5.63		up
P01023	A2MG_HUMAN	Alpha-2-macroglobulin	163.289	5.98		up
P06733	ENOA_HUMAN	Alpha-enolase	47.170	6.99		up
P06727	APOA4_HUMAN	Apolipoprotein A-IV	45.399	5.18		up
P53396	ACLY_HUMAN	ATP-citrate synthase	120.842	6.95		up
P19835	CEL_HUMAN	Bile salt-activated lipase	79.667	5.08		up
P25774	CATS_HUMAN	Cathepsin S	37.495	7.64		up
P0C0L5	CO4B_HUMAN	Complement C4-B	187.671	6.89		up
P21399	ACOC_HUMAN	Cytoplasmic aconitate hydratase	98.401	6.23		up
Q14195	DPYL3_HUMAN	Dihydropyrimidinase-related protein 3	61.964	6.04		up
P14625	ENPL_HUMAN	Endoplasmin	92.471	4.73		up
P49327	FAS_HUMAN	Fatty acid synthase	273.198	6.01		up
P02751	FINC_HUMAN	Fibronectin	272.312	5.31		up
P15328	FOLR1_HUMAN	Folate receptor alpha	29.818	8.32		up
P04406	G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	36.053	8.58		up
P11142	HSP7C_HUMAN	Heat shock cognate 71 kDa protein	68.807	5.37		up
P07900	HS90A_HUMAN	Heat shock protein HSP 90-alpha	84.663	4.94		up
P05546	HEP2_HUMAN	Heparin cofactor 2	57.072	6.26		up
P01857	IGHG1_HUMAN	Immunoglobulin heavy constant gamma 1 (Fragment)	43.911	8.46		up
P01860	IGHG3_HUMAN	Immunoglobulin heavy constant gamma 3 (Fragment)	41.325	8.23		up
P22897	MRC1_HUMAN	Macrophage mannose receptor 1	166.014	6.08		up

P40925	MDHC_HUMAN	Malate dehydrogenase, cytoplasmic	23.038	6.89		up
Q6UXI9	NPNT_HUMAN	Nephronectin (Fragment)	66.915	8.78		up
P59665	DEF1_HUMAN	Neutrophil defensin 1	10.201	6.54		up
P80188	NGAL_HUMAN	Neutrophil gelatinase-associated lipocalin	22.788	9.02		up
P43490	NAMPT_HUMAN	Nicotinamide phosphoribosyltransferase	55.522	6.69		up
P15531	NDKA_HUMAN	Nucleoside diphosphate kinase A (Fragment)	9.371	5.81		up
Q06830	PRDX1_HUMAN	Peroxiredoxin-1 (Fragment)	18.976	8.27		up
P30086	PEBP1_HUMAN	Phosphatidylethanolamine-binding protein 1	21.056	7.01		up
P36871	PGM1_HUMAN	Phosphoglucomutase-1	61.451	6.30		up
P00558	PGK1_HUMAN	Phosphoglycerate kinase 1	44.615	8.30		up
P05155	IC1_HUMAN	Plasma protease C1 inhibitor	59.493	5.97		up
P07602	SAP_HUMAN	Prosaposin	58.440	5.06		up
P07237	PDIA1_HUMAN	Protein disulfide-isomerase	56.784	4.69		up
P30101	PDIA3_HUMAN	Protein disulfide-isomerase A3	56.784	5.61		up
P14618	KPYM_HUMAN	Pyruvate kinase	49.898	7.95		up
P62820	RAB1A_HUMAN	Ras-related protein Rab-1A	22.172	5.93		up
P02768	ALBU_HUMAN	Serum albumin	69.366	5.67		up
P20061	TCO1_HUMAN	Transcobalamin-1	48.207	4.85		up
P29401	TKT_HUMAN	Transketolase	49.911	7.58		up
Q71U36	TBA1A_HUMAN	Tubulin alpha chain	57.730	4.94		up
Q16851	UGPA_HUMAN	UTP--glucose-1-phosphate uridylyltransferase	57.807	8.15		up
P47989	XDH_HUMAN	Xanthine dehydrogenase/oxidase	146.426	7.86		up
P06331	HV434_HUMAN	Ig-like domain-containing protein (Fragment)	12.943	9.01	up	
P01591	IGJ_HUMAN	Immunoglobulin J chain	18.098	4.59	up	
B9A064	IGLL5_HUMAN	Immunoglobulin lambda-like polypeptide 5	23.150	9.03	up	
P24821	TENA_HUMAN	Tenascin	240.85	4.79	up	
P07996	TSP1_HUMAN	Thrombospondin-1	129.381	4.70	up	
Q96DA0	ZG16B_HUMAN	Zymogen granule protein 16 homolog B	22.739	5.39	up	

a. theorical value

Supplementary Table 4. Differentially abundant proteins in human milk, in female and male groups, where “up” corresponds to “upregulated protein”

UniProt	ID	Protein name	MW (kDa)[a]	pI[a]	Females	Males
P63104	1433Z_HUMAN	14-3-3 protein zeta/delta	15.697	4.73		up
P23396	RS3_HUMAN	40S ribosomal protein S3	26.688	9.68		up
P06733	ENOA_HUMAN	Alpha-enolase	47.170	6.99		up
P00709	LALBA_HUMAN	Alpha-lactalbumin	16.225	4.70		up
P02647	APOA1_HUMAN	Apolipoprotein A-I	30.778	5.56		up
P06727	APOA4_HUMAN	Apolipoprotein A-IV	45.399	5.18		up
P61769	B2MG_HUMAN	Beta-2-microglobulin	11.732	6.06		up
P05814	CASB_HUMAN	Beta-casein	25.381	5.33		up
P19835	CEL_HUMAN	Bile salt-activated lipase	79.667	5.08		up
P10909	CLUS_HUMAN	Clusterin	52.495	5.88		up
P01024	CO3_HUMAN	Complement C3	187.149	6.02		up
P0C0L5	CO4B_HUMAN	Complement C4-B	187.671	6.89		up
P21399	ACOC_HUMAN	Cytoplasmic aconitate hydratase	98.401	6.23		up
Q14195	DPYL3_HUMAN	Dihydropyrimidinase-related protein 3	61.964	6.04		up
P14625	ENPL_HUMAN	Endoplasmin	92.471	4.73		up
P27105	STOM_HUMAN	Erythrocyte band 7 integral membrane protein	31.732	7.71		up
P19440	GGT1_HUMAN	Glutathione hydrolase 1 proenzyme	61.410	6.65		up
P04406	G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	36.053	8.58		up
Q9H4G4	GAPR1_HUMAN	Golgi-associated plant pathogenesis-related protein 1	14.212	9.44		up
P00738	HPT_HUMAN	Haptoglobin	45.204	6.13		up
Q96S86	HPLN3_HUMAN	Hyaluronan and proteoglycan link protein 3	47.756	6.08		up
P40925	MDHC_HUMAN	Malate dehydrogenase, cytoplasmic	23.038	6.89		up
Q6UXI9	NPNT_HUMAN	Nephronectin (Fragment)	66.915	8.78		up
P43490	NAMPT_HUMAN	Nicotinamide phosphoribosyltransferase	55.522	6.69		up
Q9H173	SIL1_HUMAN	Nucleotide exchange factor SIL1	52.085	5.22		up
P23284	PPIB_HUMAN	Peptidyl-prolyl cis-trans isomerase B	23.743	9.25		up

P05155	IC1_HUMAN	Plasma protease C1 inhibitor	59.493	5.97		up
P07602	SAP_HUMAN	Prosaposin	58.44	5.06		up
P07237	PDIA1_HUMAN	Protein disulfide-isomerase	56.784	4.69		up
P62820	RAB1A_HUMAN	Ras-related protein Rab-1A	22.172	5.93		up
Q6X4U4	SOSD1_HUMAN	Sclerostin domain-containing protein 1	23.306	9.93		up
P02768	ALBU_HUMAN	Serum albumin	69.366	5.67		up
P20061	TCO1_HUMAN	Transcobalamin-1	48.207	4.85		up
P01023	A2MG_HUMAN	Alpha-2-macroglobulin	163.289	5.98	Up	
P01860	IGHG3_HUMAN	Immunoglobulin heavy constant gamma 3 (Fragment)	41.325	8.23	Up	
O15232	MATN3_HUMAN	Matrilin-3	52.816	5.99	Up	
P05164	PERM_HUMAN	Myeloperoxidase	83.871	9.19	Up	
P59665	DEF1_HUMAN	Neutrophil defensin 1	10.201	6.54	Up	
P80188	NGAL_HUMAN	Neutrophil gelatinase-associated lipocalin	22.788	9.02	Up	
O75888	TNF13_HUMAN	Protein TNFSF12-TNFSF13	36.588	9.79	Up	
P14618	KPYM_HUMAN	Pyruvate kinase	49.898	7.95	Up	
O00300	TR11B_HUMAN	Tumor necrosis factor receptor superfamily member 11B	46.026	8.71	Up	

a. theorical value

Supplementary Table 5. Interaction networks in human milk for females

Nodes	Protein name
A2M	Alpha-2-macroglobulin
AZU1	Azurocidin
CTSG	Cathepsin G
DEFA1B	Defensin, alpha 1B
ELANE	Neutrophil elastase
ENO1	Alpha-enolase
ENO3	Beta-enolase
GP1	Glucose-6-phosphate isomerase
LCN2	Neutrophil gelatinase-associated lipocalin
MATN3	Matrilin-3
MPO	Myeloperoxidase
ORC1	Origin recognition complex subunit 1
PKM	Pyruvate kinase PKM
PRTN3	Myeloblastin
SERPINA1	Alpha-1-antitrypsin
TNFRSF11	Tumor necrosis factor receptor superfamily member 11B
TNFSF11	Tumor necrosis factor ligand superfamily member 11
TNFSF13	Tumor necrosis factor ligand superfamily member 13

Supplementary Table 6. Interaction networks in human milk for males

Nodes	Protein name
ACO1	Cytoplasmic aconitate hydratase
ALB	Serum albumin
APOA1	Apolipoprotein A-I
APOA4	Apolipoprotein A-IV
B2M	Beta-2-microglobulin
C3	Complement C3
C4B	Complement C4-B
CEL	Bile salt-activated lipase
CLU	Clusterin
CSN2	Beta-casein
DPYSL3	Dihydropyrimidinase-related protein 3
ENO1	Alpha-enolase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GGT1	Glutathione hydrolase 1 proenzyme
GLIPR2	Golgi-associated plant pathogenesis-related protein 1
HAPLN3	Hyaluronan and proteoglycan link protein 3
HP	Haptoglobin
HSP90B1	Endoplasmin
LALBA	Alpha-lactalbumin
MDH1	Malate dehydrogenase
NAMPT	Nicotinamide phosphoribosyltransferase
NPNT	Nephronectin
P4HB	Protein disulfide-isomerase
PPIB	Peptidyl-prolyl cis-trans isomerase B

PSAP	Prosaposin
RAB1A	Ras-related protein Rab-1A
RPL3	60S ribosomal protein L3
RPL4	L ribosomal proteins
RPL6	60S ribosomal protein L6
RPL8	60S ribosomal protein L8
RPS11	Ribosomal protein S11
RPS14	Ribosomal protein S14
RPS16	Ribosomal protein S16
RPS2	Ribosomal protein S2
RPS3	40S ribosomal protein S3
RPS4X	Ribosomal protein S4
RPS5	Ribosomal protein S5
SERPING1	Plasma protease C1 inhibitor
SIL1	Nucleotide exchange factor SIL1
SOSTDC1	Sclerostin domain-containing protein 1
STOM	Erythrocyte band 7 integral membrane protein
TCN1	Transcobalamin-1
YWHAZ	14-3-3 protein zeta/delta

Supplementary Table 7. Interaction networks in human milk for colostrum phase

Nodes	Protein name
ACAN	Aggrecan core protein
CD47	Leukocyte surface antigen CD47
CD79A	B-cell antigen receptor complex-associated protein alpha chain
FN1	Fibronectin type III domain containing
HGF	Hepatocyte growth factor
IGJ	Immunoglobulin J chain
IGLL5	Immunoglobulin lambda like polypeptide 5
MMP9	Matrix metalloproteinase-9
TGFB1	Transforming growth factor beta-1
THBS1	Thrombospondin-1
TIMP1	Metalloproteinase inhibitor 1
TNC	Tenascin
VEGF4	Vascular endothelial growth factor A
VWF	Von Willebrand factor
ZG16B	Zymogen granule protein 16B

Supplementary Table 8. Interaction networks in human milk for mature phase

Nodes	Protein name
A1BG	Alpha-1B-glycoprotein
A2M	Alpha-2-macroglobulin
ACLY	ATP-citrate synthase
ACO1	Cytoplasmic aconitate hydratase
ACTG1	Actin, cytoplasmic 2
ALB	Serum albumin
APOA4	Apolipoprotein A-IV
C4B	Complement C4-B
CEL	Bile salt-activated lipase
CTSS	Cathepsin S
DEFA1B	Defensin, alpha 1B
DPYSL3	Dihydropyrimidinase-related protein 3
ENO1	Alpha-enolase
FASN	Fatty acid synthase
FN1	Fibronectin type III domain containing
FOLR1	Folate receptor alpha
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIG25	Serpin peptidase inhibitor
HSP90AA1	Heat shock protein HSP 90-alpha
HSP90B1	Endoplasmin
HSPA8	Heat shock cognate 71 kDa protein
LCN2	Neutrophil gelatinase-associated lipocalin
MDH1	Malate dehydrogenase, cytoplasmic
MRC1	Macrophage mannose receptor 1
NAMPT	Nicotinamide phosphoribosyltransferase
NME1	Nucleoside diphosphate kinase A

NPNT	Nephronectin
P4HB	Protein disulfide-isomerase
PDIA3	Protein disulfide-isomerase A3
PEBP1	Phosphatidylethanolamine-binding protein 1
PGK1	Phosphoglycerate kinase 1
PGM1	Phosphoglucomutase-1
PKM	Pyruvate kinase PKM
PRDX1	Peroxiredoxin-1
PSAP	Prosaposin
RAB1A	Ras-related protein Rab-1A
RPL3	60S ribosomal protein L3
RPL4	L ribosomal proteins
RPL6	60S ribosomal protein L6
RPL8	60S ribosomal protein L8
RPS11	Ribosomal protein S11
RPS14	Ribosomal protein S14
RPS16	Ribosomal protein S16
RPS2	Ribosomal protein S2
RPS3	40S ribosomal protein S3
RPS4X	Ribosomal protein S4, X-linked
RPS5	Ribosomal protein S5
SERPIND1	Heparin cofactor 2
SERPING1	Plasma protease C1 inhibitor
TCN1	Transcobalamin-1
TKT	Transketolase
TUBA1A	Tubulin alpha-1A chain
UGP2	UTP--glucose-1-phosphate uridylyltransferase
XDH	Xanthine dehydrogenase/oxidase
YWHAZ	14-3-3 protein zeta/delta