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DIELSON DA SILVA VIEIRA

Transição do colostro para o leite de cabras: novos horizontes nos estudos das proteínas aos metabólitos

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Tese apresentada à Faculdade de Medicina Veterinária de Araçatuba da Universidade Estadual Paulista “Júlio de Mesquita Filho” – UNESP, como parte dos requisitos para a obtenção do título de Doutor em Fisiopatologia Médica e Cirúrgica.

Orientador: Prof. Adjunto Francisco Leydson Formiga Feitosa
Coorientador: Prof. Adjunto Pedro de Magalhães Padilha
Coorientador: Prof. Adjunto Mateus Matiuzzi da Costa

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DADOS CURRICULARES

DIELSON DA SILVA VIEIRA – Nascido em 10 de Junho de 1990, na cidade de Juazeiro, Bahia. Médico Veterinário formado pela Universidade Federal do Vale do São Francisco, Petrolina - PE (UNIVASF). Durante a sua graduação desenvolveu pesquisas nos âmbitos de nutrição animal, diagnóstico por imagem e microbiologia. Foi aluno da graduação em História pela Universidade de Pernambuco, Petrolina – PE (UPE), aonde participou como membro da revista acadêmica “Historien” por cinco anos. Mestre em Ciência Animal Tropical pela Universidade Federal Rural de Pernambuco, Recife – PE (UFRPE). Atuou em 2016 como médico veterinário do centro de controle de zoonoses de Petrolina – PE (CCZ/PE). Doutorando na área de Fisiopatologia médica e cirúrgica, com ênfase em Fisiopatologia médica e cirúrgica de grandes animais, no programa de Pós-Graduação em Ciência Animal da Faculdade de Medicina Veterinária (FMVA) - UNESP Araçatuba, sob orientação do Professor Adjunto Francisco Leydson Formiga Feitosa, com bolsa Fapesp (Processo Fapesp 2017/22041-1), coorientação do Professor Adjunto Pedro de Magalhães Padilha (IB/UNESP) e coorientação do Professor Adjunto Mateus Matiuzzi da Costa (UNIVASF). Durante seu doutorado, realizou período de estágio internacional de doutorado (BEPE, processo Fapesp 2019/02781-6) no departamento de bioquímica da University of Nebraska-Lincoln, na Core Facilities de Proteômica e Metabolômica sob orientação do Dr. Jiri Adamec e da Dra. Camila Braga. Participou de eventos nacionais e internacionais com apresentação de trabalhos derivados de suas pesquisas.

Dedico

Aos meus pais Janilda Vieira e José Vieira, que estiveram e sempre estarão comigo.
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Poderia haver maior milagre do que olharmos com os olhos do outro por um instante?

Henry David Thoreau

VIEIRA, D. S. **Transição do colostro para o leite de cabras: novos horizontes nos estudos das proteínas aos metabólitos.** 2020. **180 f.** Tese (Doutorado) - Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, 2020.

RESUMO

O leite de cabra é um alimento nutricionalmente complexo e que ao apresentar vários constituintes com diferentes potenciais bioativos, estes podem ser afetados por algumas enfermidades, mas que quando não, o consumo do leite e desses compostos afetam positivamente no desenvolvimento humano e animal. Assim, o objetivo deste estudo foi investigar o proteoma e metaboloma do leite caprino em diferentes estágios de lactação, idade e padrões de saúde (animais cronicamente positivos para o vírus da artrite encefalite caprina-CAEV) assim como avaliar perfis *in sílico* da modelagem da lactoferrina do leite de diferentes mamíferos, contribuindo com estudos sobre o leite de ruminantes e outros animais. Para obtenção dos resultados foram utilizadas técnicas como 2D-PAGE, espectrometria de massas e *modelagem in sílico* com programas como o I-TASSER, ExpasyProtParam, COFACTOR, entre outros. Nos estudos proteômicos e metabolômicos os animais foram divididos em grupos, sendo no mínimo sete e cinco animais por grupo, respectivamente. Diante da série de metodologias aplicadas nesse estudo, chegou-se aos resultados seguintes. Quando avaliado o proteoma da transição do colostro ao leite das cabras, observou-se uma maior qualidade nas proteínas nos animais que tiveram o primeiro parto, animais mais velhos na produção apresentaram as mesmas proteínas, porém com menor qualidade. Assim como, com três dias após o parto o padrão de concentração das proteínas também mudou, havendo de modo geral, menor concentração da proteína comparado com o dia do nascimento. Em se tratando dos metabólitos, animais mais jovens apresentaram maior quantidade de metabólitos do que os mais velhos, e não existiu diferença no perfil de metabólitos entre animais positivos e negativos para o vírus da CAE. Grupos de metabólitos foram identificados como ácidos graxos (ácido oleico, ácido palmítico etc.), açúcares (sucrose, D-maltose etc.), aminoácidos e até outros compostos orgânicos (riboflavina). Quando se avaliou a modelagem da lactoferrina, essa proteína contém sua estrutura conservada no genoma de diversas espécies de mamíferos, porém a estrutura da proteína da cabra detém uma adição de aminoácidos que não afeta a função ou aspectos biológicos dessa proteína. Os modelos ao serem gerados, foram estatisticamente validados de acordo com os parâmetros estabelecidos pelos programas utilizados, por mais que os modelos gerados da lactoferrina nas espécies *Capra hircus* e *Macaca mulatta* possuam adição de alguns grupos de aminoácidos, não há alteração no perfil

biológico e químico dessas proteínas quando comparadas com outros mamíferos. Desse modo, os dados obtidos fornecem informações acerca do proteoma e metaboloma do leite de cabras, assim como renovam informações sobre a modelagem de proteínas presentes nas secreções lácteas, contribuindo ainda mais para o crescimento de dados na pecuária de cabras de leite e estudos sobre vírus da artrite encefalite caprina.
Palavras-chave: Leite de cabra. Neonatologia. Artrite encefalite caprina. in silico. Dinâmica molecular.

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ABSTRACT

Goat's milk is a portion of nutritionally complex food and that by having several constituents with different bioactive potentials, they can be affected by some diseases, but when not, the consumption of milk and these compounds positively affect human and animal development. Thus, this study aimed to investigate the proteome and metabolome of goat's milk at different stages of lactation, age, and health standards (animals chronically positive for the caprine arthritis encephalitis virus-CAEV) as well as to evaluate in silico profiles of lactoferrin modeling from the milk of different mammals, contributing to studies on the milk of ruminants and other animals. To obtain the results, techniques such as 2D-PAGE, mass spectrometry, and *in silico* modeling were used with programs such as I-TASSER, Expasy ProtParam, COFACTOR, among others. In proteomic and metabolomic studies, animals were divided into groups, with at least seven and five animals per group, respectively. When the proteome of the transition from colostrum to the milk of goats was evaluated, a higher quality of proteins was observed in the animals that had their first calving, older animals in the production had the same proteins, but with lower quality. Likewise, with three days after delivery, the pattern of protein concentration has also changed, with, in general, less protein concentration compared to the day of birth. In the case of metabolites, younger animals had a higher number of metabolites than the older ones, and there was no difference in the metabolite profile between positive and negative animals for the CAE virus. Groups of metabolites have been identified as fatty acids (oleic acid, palmitic acid, etc.), sugars (sucrose, D-maltose, etc.), amino acids, and even other organic compounds (riboflavin). When the lactoferrin modeling was evaluated, this protein has its structure conserved in the genome of several mammal species, however, the structure of the goat protein has an addition of amino acids that do not affect the function or biological aspects of this protein. After model's generation, they were statistically validated according to the parameters established by the programs used, and the models generated from lactoferrin in the species *Capra hircus* and *Macaca mulatta* have the addition of some groups of amino acids, there is no change in the biological and chemical profile of these proteins when compared to other mammals. In this way, the data obtained provide information about the proteome and metabolome of goat's milk, as well as renew information about the modeling of proteins present in milk secretions, further contributing to the growth of data in dairy goat livestock and virus studies of goat encephalitis arthritis.

Keywords: Goat milk. Neonatology. Caprine encephalitis arthritis. in silico. Molecular dynamics.

SUMÁRIO

1 INTRODUÇÃO

O leite detém um importante papel na dieta ofertando uma gama de nutrientes necessários para o crescimento e desenvolvimento de humanos e animais. Pesquisas nos últimos 30 a 40 anos mostraram que leite e produtos lácteos podem ir além, em ajudar a reduzir o risco de doenças crônicas, incluindo osteoporose, hipertensão, e certos tipos de câncer (Huth et al., 2006; Dimenstein et al., 2010; Slacanac et al., 2010; Spertino et al., 2012; Li-Chan, 2015). Existem várias espécies animais que produzem esse rico alimento nutricionalmente e biologicamente, como vacas e cabras. No caso das últimas, são destaque especial em países em desenvolvimento com regiões de climas tropicais e subtropicais (Lobo et al, 2017).

Dentro desse cenário, a produção leiteira do Brasil aproxima-se das 35 mil toneladas, diferente de regiões como Argentina e Colômbia, que chegam a 11 mil e 6 mil toneladas, respectivamente (FAO, 2020). Mesmo diante de um sólido mercado para produtos derivados de leite bovino, a região Sudeste é a segunda maior consumidora de produtos lácteos derivados do leite de cabra, demonstrando a importância da cultura leiteira de pequenos ruminantes, não somente para o Nordeste, mas para todo o Brasil.

No Brasil, a maior parte da produção de leite caprino vem do Nordeste e do Sudeste (Lobo et al., 2017). Quanto à maior taxa de consumo, os estados do Sul e Sudeste se destacam. Uma das vantagens do leite caprino é a alta quantidade de vitamina A e seus derivados na composição (Gutiérrez-Peña et al., 2018). Também contém maior porcentagem de ácidos graxos de cadeia curta e média (C6-C14) do que o leite de vaca, contribuindo para uma coalhada mais macia do que a presente no leite bovino (Kondyli et al., 2007). Além disso, é muito indicado em casos de alergias, pois garante melhor digestibilidade e o tamanho de suas partículas gera menos estímulos alérgicos do que o leite bovino.

Vários fatores podem influenciar a qualidade e as características próprias e características do leite, como a presença de microrganismos patogênicos. O vírus da artrite encefalite caprina (CAEV) influencia a de caprinos e ovinos. Animais acometidos por essa doença podem sofrer diminuição do período de lactação e queda na qualidade do leite e dos níveis de gordura e proteínas (Smith, Cutlip, 1988; Grenwood et al., 1995).

Para entender melhor sobre o leite dos ruminantes e suas proteínas e metabólitos, assim como o efeito de doenças como a CAEV, tecnologias foram desenvolvidas com o passar dos anos (Cunsolo et al., 2015; Zhang et al., 2015). Além disso, estudos *in silico* foram desenvolvidos, tanto para implementar dados sobre o referido produto, quanto para gerar informação para a indústria e a medicina veterinária.

Em sua totalidade, a proteômica do abrange a identificação, a caracterização e a quantificação de proteínas (Zhang et al., 2011). Portanto, as aplicações buscam entender a composição básica das proteínas do leite até suas modificações pós-traducionais (PTMs), ocorrendo naturalmente ou via processamento e armazenamento do alimento (Le et al., 2017). Assim como a proteômica, a metabolômica ajuda a complementar questões sobre a biologia dos animais em geral. Essas tecnologias, contribuem com uma descrição analítica de amostras biológicas complexas e tem como objetivo caracterizar e quantificar todas as moléculas pequenas dessa amostra (Nicholson, Lindon, 2008).

Estudos sobre proteômica e metabolômica a cada dia estão fazendo-se necessários dentre os grupos de pesquisa. Ao analisar a proteômica do leite de cabras, Cunsolo et al. (2015) determinaram que ainda faltam muitos dados, para que seja possível entender mais sobre o proteoma do leite desses animais. Steinshamn et al. (2014) ao estudarem por meio da metabolômica (GC-MS) do leite de cabras alimentadas em diferentes formas de pasto, observaram que o perfil de metabólitos não se alterava, no caso mais especificamente, ácidos graxos. Ao serem usadas essas tecnologias produzem informações que cada vez mais precisam ser trabalhadas e refinadas, levando aos estudos *in sílico*.

Nos casos de estudos com modelagem de proteínas, sabe-se que estes seguem abordagem mais direcionada, empregando bioinformática, também conhecida como análise *in silico*, sem consumo de reagentes químicos, de menor custo e maior efetividade (Dziuba e Dziuba, 2010; Liu et al., 2019). Faz-se uso de métodos computacionais para gerenciar, curar e interpretar informações de sistemas biológicos, visando à descoberta mais eficiente de proteínas com potencial biotecnológico (Li-Chan, 2015). Dessa forma, este estudo buscou avaliar (i) o uso de 2D-PAGE para a identificação do proteoma do leite caprino em animais com diferentes aspectos de raça, idade e padrões de saúde (animais CAEV positivos) (ii) o metaboloma do leite caprino (dos mesmos animais do estudo sobre o proteoma) e (iii) perfis *in silico* da modelagem da lactoferrina do leite de diferentes mamíferos, contribuindo com estudos sobre o leite de ruminantes e outros animais.

1.1 Revisão de literatura

1.1.1 O consumo do leite e seus benefícios

Atualmente, há uma crescente demanda por produtos mais saudáveis, o que está gerando novo interesse pelo leite de diferentes espécies, como ovelhas e cabras, uma vez que o leite bovino representa a fonte de leite mais consumida no mundo (Caboni et al., 2018). Esse produto é uma boa fonte de proteínas (aminoácidos essenciais), gorduras (ácidos graxos insaturados), vitaminas e minerais, porém o valor nutricional dos alimentos não depende apenas do conteúdo dos nutrientes, mas também da biodisponibilidade e da contribuição desses nutrientes na ingestão diária recomendada, inclusive para a alimentação de mamíferos jovens e adultos (Zhang et al., 2011; Claeys et al., 2013; Cunsolo et al., 2015).

O conteúdo total de vitaminas no leite é altamente variável e depende da qualidade das vitaminas e do regime alimentar da fêmea (com o nível de vitaminas hidrossolúveis sendo mais influenciado pelos alimentos que o nível das vitaminas lipossolúveis). O teor de vitaminas no leite de equinos e asininos é, em média, menor que o teor de vitamina no leite dos ruminantes (Claeys et al., 2014). Por exemplo, os leites de cabra, búfalo e ovelha são excelentes fontes de vitamina A, e seu consumo contribui para a saúde devido às suas propriedades funcionais (Dimenstein et al., 2010). Isso se deve ao fato de que esses animais possuem mecanismos biológicos que convertem beta-caroteno em vitamina A com maior facilidade em relação a outros ruminantes (Abd El-Salam, El-Shibiny, 2011).

Os glóbulos de gordura do leite de cabra são menores que os do leite de vaca, o que lhe confere melhor digestibilidade. Suas características nutricionais e a alcalinidade distinta contribuem para sua recomendação na dieta infantil e de idosos (Dimenstein et al., 2010; Roncada et al., 2012), sendo um produto recomendado para as demandas do atual mercado consumidor de leite no Brasil.

Segundo estudo desenvolvido por Bindal & Wadhwa (1993), os níveis de ácidos graxos de cadeia curta (SCF) e ácidos graxos de cadeia média (MCF) na manteiga de cabra foram significativamente maiores do que no produto derivado dos leites de vaca e búfala, assim como os ácidos C 10:0 e C 12:0 no produto de cabra estavam cerca de 2-3 vezes mais presentes do que no dos outros dois animais. Os mesmos autores relatam que essas características influenciam no sabor do produto derivado do leite, considerando que o leite de cabra contém um sabor mais particular do que os produtos derivados dos leites de vaca e búfala. Sun et al. (2020) acrescentam que as partículas de gordura do leite são capazes de guardar informações

que possivelmente também serão absorvidas pelo organismo. O conteúdo de proteínas no leite e na gordura desse produto são fatores indicativos de sua qualidade. Cerca de 5% das proteínas com potencial benéfico à saúde animal e humana se encontram na camada denominada membrana do glóbulo de gordura do leite (MFGM).

Com o intuito de entender mais sobre os glóbulos de gordura do leite dos ruminantes. Os precursores dos MFGM são formados no retículo endoplasmático das células epiteliais da glândula mamária sendo liberados no citosol como gotículas lipídicas rodeadas por um revestimento de monocamada de proteínas e fosfolipídios (Spertino et al., 2012), junto a esses componentes, inúmeras informações e produtos, como proteínas e metabólitos. Assim, as proteínas presentes na MFGM são capazes de estimular a atividade celular, promovendo os processos de crescimento e defesa, como observado em estudo com células Caco2, que são linhas celulares do adenocarcinoma colorretal (Spertino et al., 2012). Devido a sua estrutura, a MFGM protege e viabiliza os compostos bioativos e nutrientes de maneira eficiente para o neonato (Spertino et al., 2012).

Dessa forma, é preciso observar, também, que mesmo em outros componentes do leite há diferenças. As funções associadas à síntese de proteínas são mais abundantes no colostro do que no leite, assim como o leite aparenta possuir mais proteínas relacionadas ao metabolismo (Sun et al., 2020). Todos os fatores relatados demonstram que o leite contém componentes que podem, seguir vias dentro organismos e beneficiá-los após ingeridos, sejam de animais ou humanos.

1.1.1.1 Importância para os neonatos ruminantes

O leite fornece todos os nutrientes essenciais, substratos e moléculas que favorecerão o crescimento e a saúde do recém-nascido; além disso, está documentado que o leite foi evolutivamente selecionado devido à sua capacidade de proteger os neonatos de patógenos bacterianos (Ozturk et al., 2019). Dessa forma, sua ingestão é importante para a transferência de imunidade contra patógenos, da mãe para o recém-nascido, por conter proteínas antimicrobianas e imunomoduladoras, que estarão ativas no sistema do neonato quando este consumir o colostro/leite (Cunsolo et al., 2015).

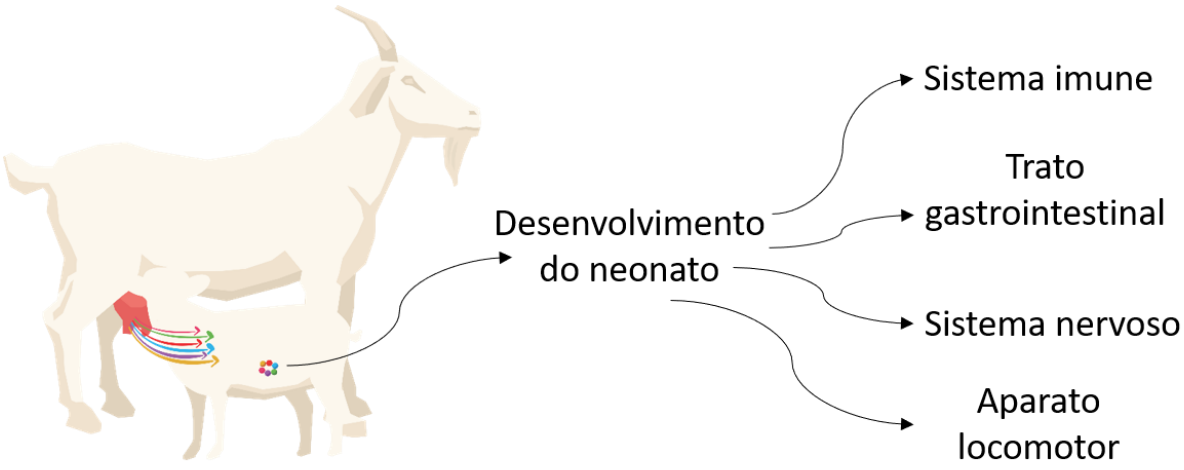
O colostro e o leite são importantes para os ruminantes, devido às diferenças de placentação entre ruminantes e primatas (ou seja, implantação tardia *versus* precoce; placenta epiteliocorial *versus* hemocorial em primatas e ruminantes, respectivamente), fazendo com que esses animais sejam utilizados como modelo para processos imunológicos durante a gestação (Morel et al., 2012; Moffett, Loke, 2006). Mais precisamente, os ruminantes possuem placenta sinepiteliocorial, na qual as células binucleadas do trofectoderma migram, invadem e se fundem

com o lúmen uterino para formar sincícios multinucleados em áreas aglandulares do útero onde os placentomas se formam. Nesse tipo de placentação epiteliocorial/sinepiteliocorial, o concepto permanece dentro do lúmen uterino ao longo da gestação (Bazer et al., 2012).

Devido a característica da placenta e outras questões evolutivas, o consumo dos produtos lácteos (colostro e leite) após o nascimento é um fator determinante à sobrevivência dos neonatos ruminantes. Ambos apresentam alterações em sua composição, sendo elas características físicas e funcionais (Tsioulpas et al., 2007; Abd El-Fattah et al., 2012). O colostro é uma das mais ricas fontes de imunoglobulinas para o recém-nascido e sua ingestão deve garantir o que se denomina “imunidade passiva” para sobreviver aos primeiros dias de vida. O não consumo desse produto nas primeiras horas de vida pode levar ao risco de falha na transferência imunológica passiva (TIP), aumentando as taxas de morbidade e mortalidade (O'Brien, Sherman, 1993, Argüello et al., 2004).

Centenas de proteínas já foram identificadas no leite e reconhecidas com diversas funções biológicas (Zhang, et al., 2016). Por exemplo, as proteínas presentes no soro do leite contribuem para o desenvolvimento do sistema imunológico e, devido às variações que ocorrem entre as espécies, é possível compreender que o leite de diferentes espécies fornece proteção para os recém-nascidos de várias maneiras (Figura 1), além de contribuírem com cerca de 20% do conteúdo de proteínas presentes na secreção láctea (Beck, et al., 2015; Lu et al., 2018).

Figura 1 - Esquema do reflexo do consumo do colostro/leite no organismo do neonato ruminante.



Fonte: Elaborado pelo autor

Assim, faz-se necessária a comparação entre proteomas de várias espécies de mamíferos, pois as informações sobre o leite desses animais podem servir como suporte de dados para o uso de diferentes fontes de leite como base material para fórmulas simplificadas

e descobrir ingredientes biológicos ativos de fontes alternativas de leite que podem ser adicionadas às infantis (Lu et al., 2018).

Um estudo metabolômico realizado em leite de cabra mostrou que este apresenta um teor maior de mannose-6-fosfato e de fucose do que o leite ovino, e que esses açúcares são benéficos para o desenvolvimento do ambiente gastrointestinal de neonatos (Caboni et al., 2018). É possível também encontrar uma variedade maior de oligossacarídeos no leite de cabra, em comparação com o leite bovino. Sabe-se que esses compostos estimulam o crescimento de microrganismos benéficos no cólon dos neonatos, atuam como análogos de receptores que inibem a ligação de microrganismos patogênicos à mucosa do cólon e pequenas quantidades são absorvidas pela circulação, onde modulam aspectos imunológicos (Urashima et al., 2013).

Pesquisar sobre o metaboloma do leite de cabra é importante para produzir mais dados científicos sobre este complexo alimento, visto que até 2017 existiam apenas alguns estudos (Park et al., 2007; Steinshamn et al., 2014) usando essa tecnologia em leite/colostro (Goldansaz et al., 2017). Goldansaz et al. (2017) descreveram que espécies como caprinos, ovinos e suínos são as menos estudadas em termos de “ômicas” do grupo de animais de produção (Goldansaz et al., 2017). Portanto, estudos sobre a transição do colostro ao leite e a utilização de técnicas “ômicas” são necessários para ajudar a entender mais a respeito de um produto tão rico e que influencia positivamente o desenvolvimento dos ruminantes e o mercado e nutrição humano.

1.1.2 Vírus da artrite encefalite caprina (CAEV)

As doenças infecciosas são fatores comuns na produção animal e principalmente na cultura leiteira. A mastite é a doença de produção mais estudada para a indústria de laticínios, sendo considerada a que mais traz prejuízos financeiros e perdas econômicas. Tais perdas acontecem sob a forma do descarte do leite de animais doentes clinicamente infectados, leite de animais tratados com antibióticos e o descarte e reposição de animais; além disso, os custos do tratamento e acompanhamento veterinário contribuem para que isso ocorra (Akers, Nickerson, 2011).

De fato, um único quarto infectado durante a lactação pode reduzir a produção de leite de uma vaca em 10 a 12% e o monitoramento ocorre principalmente pela contagem de células somáticas (CCS) (Akers, Nickerson, 2011). A glândula mamária sofre alterações durante estágios do processo inflamatório, causando o aumento dessas células e, para a maioria dos laticínios, o controle da mastite é realizado pela CCS, avaliação do teor de proteínas, gordura e do crescimento microbiano (Akers, Nickerson, 2011).

Além de doenças como a mastite, que comumente afetam ruminantes em grande número, existem doenças como a artrite encefalite caprina (AEC), que é causada por vírus. Os lentivírus de pequenos ruminantes (SRLV) são retrovírus que pertencem ao subgrupo lentivírus, que inclui ainda os vírus da imunodeficiência dos símios (SIV), da imunodeficiência felina (FIV), da imunodeficiência bovina (BIV) e da anemia infecciosa equina (EIAV) (Blacklaws, Harkiss, 2010).

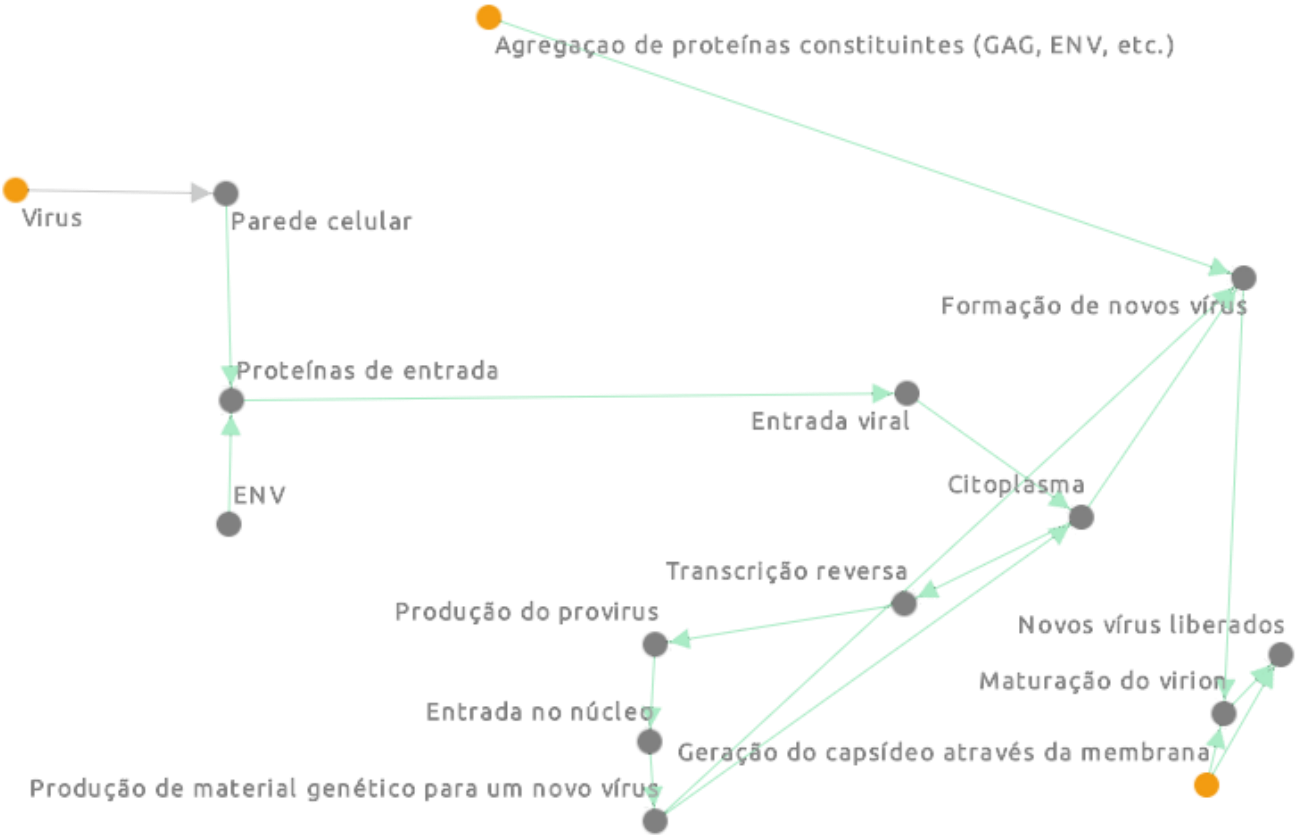
SRLV é o termo coletivo usado para o vírus maedi-visna (VMV) e o vírus da artrite encefalite caprina (CAEV). Embora o VMV e o CAEV fossem originalmente estudados como vírus separados devido às espécies que mais afetam, são vírus estreitamente relacionados, agora pertencentes a um único grupo de vírus que pode infectar ovelhas e cabras (Blacklaws, Harkiss, 2010).

Quando se discute sobre as lentiviroses, sabe-se que os anticorpos neutralizantes desempenham papel significativo no controle das infecções persistentes, assim como para o HIV; contudo, eles reagem amplamente, o que pode afetar o organismo animal ou humano (Pantophlet; Burton, 2006; West et al., 2014). O vírus da artrite encefalite caprina, membro dos lentivírus, é muito comum entre os rebanhos de caprinos e ovinos, sendo capaz de influenciar o organismo das cabras de inúmeras formas, não havendo muitos estudos direcionados à proteômica de animais acometidos por essa doença (Bezerra Júnior et al., 2018).

Quando adentram o organismo animal, os SRLV replicam produzindo um intermediário de DNA chamado provírus, que é integrado ao genoma da célula hospedeira (Blacklaws, 2012). O “vírion” que contém duas cópias do genoma viral para permitir a síntese do provírus (Figura 2) é envolvido e incorporado à sua membrana pela proteína ENV. Uma vez que o capsídeo é liberado no citoplasma da célula alvo, ocorre a transcrição reversa, assim o complexo de pré-integração é transportado para o núcleo onde o provírus será integrado ao genoma do hospedeiro (Blacklaws, 2012).

A transcrição é conduzida pelo promotor na região LTR proviral, a primeira então, é feita durante a síntese do provírus, permitindo a expressão de mRNA viral e genômica RNA; a partir disso, a proteína ENV viral é inserida nas membranas (Figura 2) das células hospedeiras, incluindo a membrana plasmática (Freed, Martin, 2007; Goff, 2007). Ocorrendo esses processos e a formação de novo material genético viral na célula hospedeira, haverá a produção de proteínas importantes para a liberação do vírus, como as proteínas (Figura 2) GAG, POL, ENV, entre outras, além de estímulos por parte do vírus (Blacklaws, 2012).

Figura 2 - Representação esquemática geral do processo de replicação viral dos lentivírus construída usando o servidor CellCollective© (Helikar et al., 2012). Os pontos amarelos são identificações de partes chaves do processo de replicação.



Fonte: Elaborado pelo autor gerado com informações fornecidas por BLACKLAWS, 2012

Com a entrada do vírus no organismo e todo o processo de replicação, uma resposta imunológica ocorre; porém, essa resposta acentuada de anticorpos é pouco neutralizante e pode contribuir para os mecanismos imunopatogênicos da artrite induzida pela CAEV, assim como causar sequelas patológicas como pneumonia, encefalite e mastite (Knowle et al., 1990; Bertoni et al., 1994). Nas últimas décadas o interesse sobre a utilização da proteômica associada aos diferentes diagnósticos se tornou muito forte em humanos, porém em doenças como a CAEV e, principalmente, em enfermidades de pequenos ruminantes, a sua utilização ainda é escassa.

Estudos sobre as mais variadas relações do lentivírus da CAEV com as fêmeas caprinas são importantes, uma vez que o vírus tem a glândula mamária como um dos órgãos de predileção para multiplicação e disseminação. A transmissão por via seminal para esses animais pode ocorrer (Souza et al., 2013). Assim, entender mais sobre o vírus e como este se dissemina no organismo dos caprinos é um desafio, necessitando-se do uso de novas ferramentas, como a proteômica e metabolômica.

1.1.3 Proteômica e metabolômica: aplicações na pecuária leiteira

Proteômica é o estudo em larga escala de proteínas, geralmente por métodos bioquímicos. Pesquisas com essa tecnologia iniciaram com a utilização de géis bidimensionais para criar bancos de dados de todas as proteínas expressas em determinados tecidos e fluidos (Pandey, Mann, 2000). Nos anos 90, a espectrometria de massas surgiu como um método analítico poderoso para estudo de funções biológicas, ajudando ainda mais as pesquisas e diminuindo as limitações da análise de proteínas (Pandey, Mann, 2000). Hoje, o termo proteômica vai muito além da identificação de proteínas. Atualmente, existe a capacidade de se avaliar de modo dinâmico os sistemas biológicos utilizando essa ferramenta.

Ao usar essa técnica, se obtém o entendimento sobre o proteoma, que corresponde ao conteúdo total de proteínas de um sistema biológico específico e, por ser altamente dinâmico, pode sofrer constantes mudanças de acordo com diferentes estímulos. A proteômica inclui o conhecimento estrutural e funcional das proteínas, a quantificação da concentração, o estudo de sua localização e modificações e as interações entre elas. Há alguns anos essa técnica estava sendo usada apenas em pesquisas biomédicas, contudo foi se revelando uma ferramenta poderosa na pesquisa em ciências de alimentos (Ortea et al., 2016).

A análise proteômica pode ser aplicada para a busca sistemática de novas proteínas/peptídeos marcadores, acelerando o desenvolvimento de ensaios para avaliação de alimentos. Adicionalmente, resultados de estudos proteômicos podem influenciar no desenvolvimento de metodologias analíticas, precisas e confiáveis para detectar as proteínas/peptídeos marcadores previamente identificados e validar a sua presença em situações biológicas e, até mesmo, de adulteração de alimentos (Ortea et al., 2016).

Eletroforese em gel 2D (2D-PAGE) e separação multidimensional cromatográfica, entre outros métodos, são frequentemente utilizados para reduzir a complexidade da amostra biológica, confluindo com a resolução de informações acerca do proteoma (Lambert et al., 2005; Malmström et al., 2006; Slebos et al., 2008). Nos estudos sobre o proteoma, a tecnologia por *shotgun* refere-se à análise direta de misturas proteicas complexas para gerar rapidamente um perfil global das proteínas nos fluidos orgânicos ou tecidos. Essa abordagem foi facilitada devido à incorporação da cromatografia líquida de alta pressão multidimensional (LC/LC), espectrometria de massa em sequência (MS/MS) e algoritmos de busca de banco de dados (Wu; MacCoss, 2002).

Embora 2D-PAGE tenha suas limitações no que diz respeito à identificação de proteínas de baixo peso molecular, proteínas de membrana, bem como proteínas com propriedades físico-químicas extremas (ou seja, em termos de peso molecular ou ponto isoelétrico), é uma

técnica muito usada e, por vezes, suficiente, no entendimento e superficial visualização do proteoma de uma amostra complexa (Lambert et al., 2005; Slebos et al., 2008).

Métodos de *shotgun* para proteômica estão rapidamente substituindo a técnica de 2D-PAGE com LC/MS, possibilitando identificar centenas e quem sabe milhares de proteínas diretamente dos lisados celulares, dependendo também do tipo de amostra, pois sua "praticidade" faz com que seja aplicada com sucesso na proteômica, incluindo caracterização de complexos proteicos, de modificações de proteínas e perfil de expressão de proteínas (Delahunty; Yates III, 2005; Zhang et al., 2013).

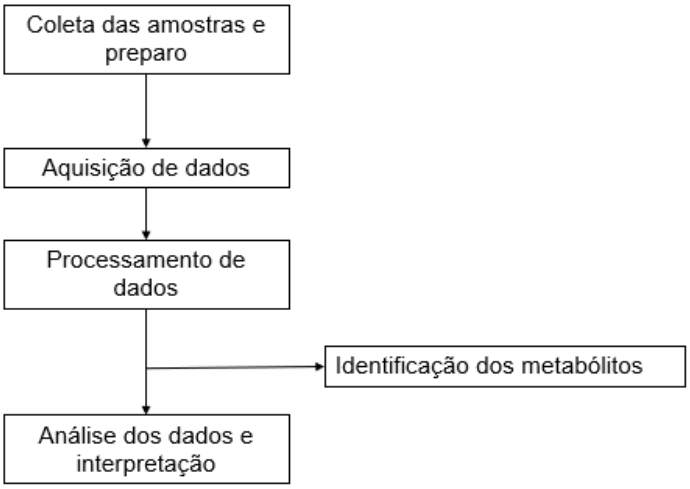
Desse modo, a estratégia *gel-free* (*shotgun*) tornou-se uma frente de estudos importante para análise de proteínas e suas modificações, podendo, portanto, contribuir ainda mais para os conhecimentos sobre a transição do colostro ao leite em cabras. Contudo, técnicas baseadas em gel continuam sendo requisitadas com o intuito de validar pesquisas utilizando técnicas mais avançadas. Assim, em se tratando de estudos com proteômica, as metodologias podem se complementar.

Avançando ainda mais em termos de entendimento sobre a complexidade de tecidos e fluidos biológicos, outras metodologias estão sendo consolidadas. As ômicas mais recentes são, naturalmente, metabolômica e lipidômica; a primeira é um campo em rápida expansão dentro das técnicas "ômicas", sendo considerada útil, já que se apresenta como uma possível complementação da genômica e da proteômica. Essa técnica vem sendo bastante utilizada nos últimos anos em estudos relacionados à autenticidade e integridade de alimentos, especialmente porque os metabólitos são frequentemente essenciais para a nutrição humana (Ellis et al., 2016).

Outro foco atual é a utilização de metabolômica em estudos com plantas medicinais, já que o isolamento de compostos de plantas é um fator importante para a indústria (Quansah, Karikari, 2016). De fato, esses recentes estudos metabolômicos parecem abranger não apenas uma gama mais ampla de métodos tecnológicos, mas também uma gama mais diversificada de aplicações nesta área (Ellis et al., 2016).

Existem várias formas de analisar e obter resultados voltados à metabolômica. Suas duas principais abordagens são a criação de perfis de metabólitos "target" (direcionados) e "untarget" (não direcionados) (Quansah, Karikari, 2016). Ao utilizar a metodologia "target", busca-se avaliar um grupo pré-determinado de metabólitos, gerando pesquisa tendenciosa a um grupo ou grupos específicos de produtos. Então, ao se escolher o método "untargeted" (Figura 3), além de se ter uma abordagem mais básica, é possível gerar avaliações qualitativas e quantitativas dos metabólitos nas amostras complexas (Chauthe et al., 2012; Commisso et al., 2013).

Figura 3 - Fluxo de trabalho básico de metodologia “untargeted” para metabolômica.



Fonte: Elaborado pelo autor e modificado a partir de QUANSAH, KARIKARI, 2016

As técnicas analíticas empregadas em estudos metabolômicos incluem cromatografia líquida com espectrometria de massa (LC/MS), cromatografia gasosa com espectrometria de massa (GC/MS), eletroforese capilar, cromatografia em camada fina, e espectrometria de ressonância magnética nuclear (RMN) (Quansah, Karikari, 2016).

Diante dessas tecnologias (proteômica e metabolômica) e da necessidade de se entender mais sobre o leite, doenças infecciosas e aspectos biológicos, muitos estudos foram realizados com o passar dos anos (Zhang et al., 2011; Cunsolo et al., 2015; Zhang et al., 2015; Thomas et al., 2016). Nesses estudos tanto tecnologias 2D-PAGE (Zhang et al., 2011) quanto a utilização diretas como a *shotgun* (Zhang et al., 2015) foram utilizadas, trazendo à luz informações sobre o leite bovino, porém cada vez mais existem estudos com o leite de cabra (Cunsolo et al., 2015; Caboni et al., 2019; Gerlando et al., 2019).

Não somente sobre o leite de animais saudáveis, muitas pesquisas foram e continuam sendo desenvolvidas para entender como doenças infecciosas causadas por microrganismos, como a mastite, podem influenciar o proteoma da glândula mamária (Boehmer et al 2010; Thomas et al., 2016; Salama et al., 2020). Também na indústria, tecnologias como proteômica e metabolômica demonstraram essenciais, aplicadas em estudos sobre o efeito do aquecimento do leite (Chen et al., 2019a), a produção de “kefir” (Izquierdo-Gonzales et al., 2019) e, até mesmo, se o processo de homogeneização do leite foi capaz de alterar a qualidade do produto (Chen et al., 2019b).

Assim, essas tecnologias demonstram-se cada vez mais importantes, ajudando no entendimento sobre um produto mundialmente consumido como o leite e confluindo em dados

que, agregados aos estudos genômicos e transcriptômicos, geram mais respostas, reunindo os organismos estudados e atribuindo mais valor ao leite.

1.1.4 Atividade antimicrobiana e modelagem de proteínas

O leite conserva componentes que inibem o crescimento microbiano e contribuem para a imunidade do recém-nascido. O fato de o leite deter compostos com atividade biológica é frequentemente utilizado como argumento para atribuir benefícios para a saúde, destacando um determinado tipo de leite ou seu consumo cru (Claeys et al., 2014). Dentre os muitos componentes do leite materno com potenciais biológicos importantes para a saúde e o desenvolvimento do recém-nascido, estão os ácidos graxos poli-insaturados de cadeia longa, os oligossacarídeos complexos e os fatores de crescimento, porém acredita-se que a maior variedade de atividades biológicas é fornecida pelas proteínas (Lönnerdal, 2017).

É possível encontrar no leite proteínas como lactoferrina (lactotransferrina), α-lactalbumina, lisozima, osteopontina, k-caseína, xantina oxidase e muitas outras (Lönnerdal, 2017; Ozturk et al., 2020). Elas possuem mecanismos de ação diferentes, todavia cada qual desempenha um importante papel na proteção e no desenvolvimento dos neonatos. Durante a alimentação, a maioria das proteínas do leite é clivada em peptídeos, que estão comumente presentes no leite ou no trato gastrointestinal (TGI) do recém-nascido, sendo essa uma fase importante no processo de amamentação. Caso ocorra algum problema durante a digestão das proteínas no TGI do recém-nascido pode haver prejuízos à quantidade de aminoácidos disponíveis para que ocorra a síntese de proteínas, mas também a liberação de peptídeos bioativos das proteínas do leite (Beverly et al., 2020).

Dentre as proteínas fundamentais aos neonatos, encontram-se a osteopontina e a lactotransferrina. A primeira está envolvida em muitos processos biológicos, incluindo a sobrevivência celular, remodelação óssea, inibição da calcificação ectópica e funções imunomodulatórias. Sendo assim, animais sem osteopontina tiveram infecções mais graves e demoraram a eliminar as micobactérias após a infecção (Nau et al., 1999).

Já a lactotransferrina (LTF) ou lactoferrina (LF) é uma glicoproteína da família das transferrinas, de 80 kDa, com dois locais de ligação ao ferro de alta afinidade. Esta proteína não se encontra apenas em fluidos exócrinos, como colostro, leite, lágrimas e saliva, mas também em leucócitos e neutrófilos polimorfonucleares. Desempenha um papel defensivo do hospedeiro, mais precisamente nas superfícies das mucosas, exibindo atividade antimicrobiana, sendo considerada uma proteína de defesa inata, muito presente no leite (Farnaud, Evans, 2003; Murata et al., 2013).

Um fato interessante sobre essa proteína é que ela pode estar complexada com outras proteínas antimicrobianas, como as imunoglobulinas (Ig), o que pode resultar no aumento da sua atividade antimicrobiana. Estes e outros efeitos são interessantes tanto na lactação quanto no período de involução da glândula mamaria, no que diz respeito à suscetibilidade desta a ataques microbianos (Wang, Hurley, 1998). Essa proteína é abundante no leite humano, totalizando aproximadamente 1-2 mg/mL, porém sua concentração no leite de ruminantes é cerca de 10 a 100 vezes menor, chegando a valores próximos de 0,02–0,2 mg/mL (Le Parc et al., 2014).

As propriedades funcionais da lactoferrina do leite de cabra são próximas às do leite humano e, portanto, essa proteína representa alternativa a produtos que podem ser consumidos por humanos, mas anteriormente seriam produzidos com proteínas derivadas do leite bovino. Devido aos seus benefícios, a lactoferrina é bastante utilizada para suplementação, devido ao fato que seu consumo isolado reflete em efeitos benéficos (Murata et al., 2013).

Buscando entender melhor sobre essas proteínas e suas aplicações industriais, ocorre a necessidade de estudos *in silico*. Mais precisamente entender por meios computacionais (modelagem), utilizando programas, como essas proteínas se apresentam sem que haja interferência de fatores biológicos. Métodos *in silico* são realizados usando computadores em vez de experimentos convencionais com materiais e, mesmo que a pesquisa experimental continue a ser a ferramenta principal e essencial para estudar compostos em alimentos, o papel das abordagens *in silico* é agora mais rotineiro e diverso, sendo bem comum seu uso para estudos relacionados com enzimas alimentares, devido ao seu desempenho rápido e imenso potencial (Zhang et al., 2019).

As moléculas com aminoácidos como parte de sua construção, como peptídeos bioativos derivados de alimentos (BPs), são um dos principais componentes presentes em alimentos que afetam a atividade funcional e biológica dos seus produtos alimentícios e derivados (Barati et al., 2020). Estudos *in silico*, então, ajudam a entender mais sobre o potencial de componentes dos alimentos como as proteínas.

Para que se possa trabalhar com modelagem de dados, é preciso uma disponibilidade de informações estruturais preliminares; por exemplo, quando estudada a proteína do choque térmico 70 (HSP70) percebeu-se que essa compartilha um alto percentual de semelhança entre vários eucariotos, o que a torna uma opção acessível para avaliar papéis funcionais usando estudos *in silico* (Singh et al., 2019). Nesse estudo foi observado que anteriormente não havia disponibilidade de estrutura experimental da proteína HSP70 para *Bubalus bubalis* (BB) e, portanto, a avaliação de HSP70 em BB tornou-se essencial para analisar o mecanismo

prospectivo de seu funcionamento em búfalos e estudos de interação com outras moléculas (Singh et al., 2019).

Desse modo, percebe-se que estudos como o realizado nessa tese abrem caminhos que poderão direcionar pesquisadores e incentivar a indústria, caso se deseje produzir ou combater a atividade de uma proteína. A lactoferrina está presente no leite não somente de humanos e ruminantes, mas de várias espécies de mamíferos, e o melhor entendimento sobre seus aspectos estruturais, químicos, físicos e biológicos, é fator determinante para que se possa conhecer mais sobre o leite.

Pesquisadores que estudam, modelagem e pesquisas *in silico* com enzimas destacam que há dificuldade para os enzimologistas de alimentos selecionarem o programa, o algoritmo e/ou parâmetros apropriados para realizar a previsão, visto que esses fatores provavelmente contribuem para resultados diferentes (Zhang et al., 2019). Zhang et al. (2019) acreditam que as tendências futuras incluem a incorporação eficiente de abordagens *in silico* e experimentais para resolver problemas antigos ou novos, e que devem ser criadas melhorias em bancos de dados de enzimas alimentares, sites, programas e ferramentas computacionais (Zhang et al., 2019).

Esses métodos se aplicam aos estudos das proteínas do leite, visto que é um material inteiramente complexo e muitos fatores podem alterar a sua composição. Contudo, ao se trabalhar com aplicações *in silico*, parte-se do princípio de que os resultados dessa pesquisa possam alavancar outras, fornecendo informações base para situações controladas, diferente de um organismo, onde desde a temperatura corporal quanto o pH do ambiente podem causar alterações.

2 OBJETIVO

Os objetivos desse estudo foram avaliar (i) o proteoma do leite caprino em diferentes estágios de lactação, idade e padrões de saúde, (ii) o metaboloma do leite caprino de acordo com a transição do colostro ao leite e padrões de saúde e (iii) perfis *in silico* da modelagem da lactoferrina do leite de diferentes mamíferos, contribuindo com estudos sobre o leite de ruminantes e outros animais.

3 CAPÍTULO 1 - 2D-PAGE ELECTROPHORESIS: UNDERSTANDING THE PROTEOME OF TRANSITION FROM COLOSTRUM TO MILK IN GOATS WITH AND WITHOUT THE ARTHRITIS ENCEPHALITIS CAPRINE VIRUS

3.1 Resumo

Nós utilizamos uma abordagem proteômica no intuito de compreender a transição de colostro ao leite em cabras saudáveis, assim como em animais cronicamente positivos para o vírus da artrite encefalite caprina (CAEV). Quatro grupos foram designados para marcar essa transição, animais de primeiro parto da raça Saanen (G1, n=7) e da raça Alpina (G2, n=7), animais negativos (G4, n=7) e positivos (G5, n=7) para o vírus da CAEV. O proteoma foi investigado por eletroforese bidimensional (2D-PAGE) e as proteínas diferencialmente expressas observadas por meio do software Progenesis Samespot. As proteínas do soro foram quantificadas usando ensaio de Biureto. Os resultados mostram que o conteúdo de proteína total diminui em cabras com infecção por CAEV. A infecção por CAEV acelera o período de transição do colostro para o leite, indicado por uma perda no teor de proteína total quando comparada com cabras saudáveis. Quando avaliada a concentração dos spots por fold change, cabras Saanen e Alpina não apresentam diferença. Os animais negativos apresentam maior quantidade de spots com maior fold change comparando-se com animais positivos para o vírus da CAE. Os resultados preliminares podem ser utilizados para subsidiar estudos sobre aspectos do leite para recém-nascidos de cabras infectadas com CAEV e/ou cabras mais velhas em fazendas leiteiras de países em desenvolvimento.

Palavras-chave: Proteína. Ruminante. Recém-nascido.

3.2 Abstract

We used a proteomic approach to understand the transition from colostrum to milk in healthy goats, as well as in chronically positive animals for the caprine arthritis encephalitis virus (CAEV). Four groups were assigned to mark this transition, first-calf animals of the Saanen breed (G1, n = 7) and the Alpine breed (G2, n = 7), negative animals (G4, n = 7), and positive animals (G5, n = 7) for the CAEV virus. The proteome was investigated by two-dimensional electrophoresis (2D-PAGE) and differentially expressed proteins observed using the Progenesis Samespot software. Whey proteins were quantified using Biuret assay. The results show that the total protein content decreases in goats with CAEV infection. When assessing the concentration of spots by fold change, Saanen and Alpine goats show no difference. Negative animals have a higher number of spots with greater fold change compared to positive animals for the CAE virus. Preliminary results can be used to support studies on aspects of milk for newborns of goats infected with CAEV and/or older goats on dairy farms in developing countries.

Keywords: Protein. Ruminant. Newborn.

3.3 Introduction[1]

Goats production is an important socio-economic activity both for the meat and milk (Vogado et al., 2017). In some countries (part of Latin America as Mexico and most of South America), dairy goats give an important contribution to smallholder livelihoods, especially in arid areas that are poorly suited for agricultural crops (Lu, Miller, 2019). Production and composition of goat milk in Brazil are influenced by genetic and non-genetic factors such as lactation order, herds, human index and geographical location (Lu, Miller, 2019). With this development of the goat culture in regions like Brazil, the control of the environment and breeding, for example, should be considered since they can affect the milk production, bring sanitary problems and diseases.

Caprine arthritis encephalitis virus (CAEV) infection affect the health of the small ruminants and that can reflect in the quality of colostrum/milk, and the direct consuming by the newborn may cause risk of infection through the virus (Tsioulpas et al. 2007; Abd El-Fattah et al. 2012). Chronic progressive arthritis of the carpus or tarsus is seen in lentivirus-infected animals, with a higher incidence in goats (Blacklaws, 2012).

To become infected via fusion between the cells, they need not have specific viral receptors and with that, the flexibility of lentiviruses in infecting cells by both classical receptor-mediated endocytosis and by fusion may permit a wider host cell tropism (Zink et al., 1990 CAEV-infected goats show a reduced cellular immunity to lentiviruses, as CAEV-infected immune systems establish a robust humoral immune response against several virus proteins, is the response diminishes over time, overloaded by a hypersensitivity (Knowle et al., 1990; Bertoni et al., 1994). However, this pronounced antibody response poorly neutralizes and may contribute to the immunopathogenic mechanisms of CAEV-induced arthritis, causing post-sequelae symptoms such as pneumonia, encephalitis, and mammary gland inflammation (Knowle et al., 1990; Bertoni et al., 1994). Still, infected animals present weight loss and malnutrition because the reflection of the virus replication in the small ruminant organism.

More studies focus on the effects of the virus replication in organs like the mammary gland of goats seems to be important. For investigate proteins, for example, aspects of diseases such as CAEV, 2D-PAGE electrophoresis is a classical method used to identify macromolecules present in colostrum and milk. This method separates large proteins by pI (isoelectric point) and a relative molecular weight (MW) from samples in differing species, organs, and fluids (Jorrin-Novo et al., 2018). Gel electrophoresis, in general, and the most employed 2-DE versions (IEF-

[1] Small Ruminant Research- ANEXO 2

SDS-PAGE, ND BN-SDS PAGE, and DIGE), in particular, have been subject to extensive reviews and discussion this technique is well accepted, and corroboration with other techniques (Jorrin-Novo et al., 2018).

During this transition from colostrum to regular milk, gradual or sudden composition and protective properties change may occur and what affect the immune response in newborn ruminants (Arain et al., 2008). It is known that milk contains essential nutrients for young mammals and a common source of proteins and vitamins and/or essential minerals for immune health for adults (Zhang et al., 2011; Cunsolo et al., 2015). There are no protein studies on the transition from colostrum to milk with such an impact in Brazil using 2D-PAGE electrophoresis, as the caprine arthritis encephalitis virus (CAEV) endemic is widespread in the herds of the mentioned species (Bezerra Júnior et al., 2018).

This kind of electrophoresis can be used as a marker for identifying changes that occur in the transition period from colostrum to milk in ruminant species. Therefore, this project aims to elucidate these mechanisms employing 2D-PAGE electrophoresis to understand the transition of the proteome from colostrum to milk in ruminants, in different breeds Saanen and Alpine goats[(i);] and Saanen goats with and without CAE virus infection[(ii).]

3.4 Materials and methods

3.4.1 Ethical and research group aspects

The study was carried out through a partnership between the Large Animal Sector of UNESP/FMVA, with the collaboration of the Bioanalytical and Metalloproteomics Laboratory (LBM/IB) of the Biosciences Institute of Unesp/ IBB and a dairy goat farm located in São José do Rio Preto/SP (20°53'26.5"S 49°20'41.8"W) and counted with the ethical approval of the Ethics Committee on Animal Experimentation (CEUA – FOA number 00422-2018).

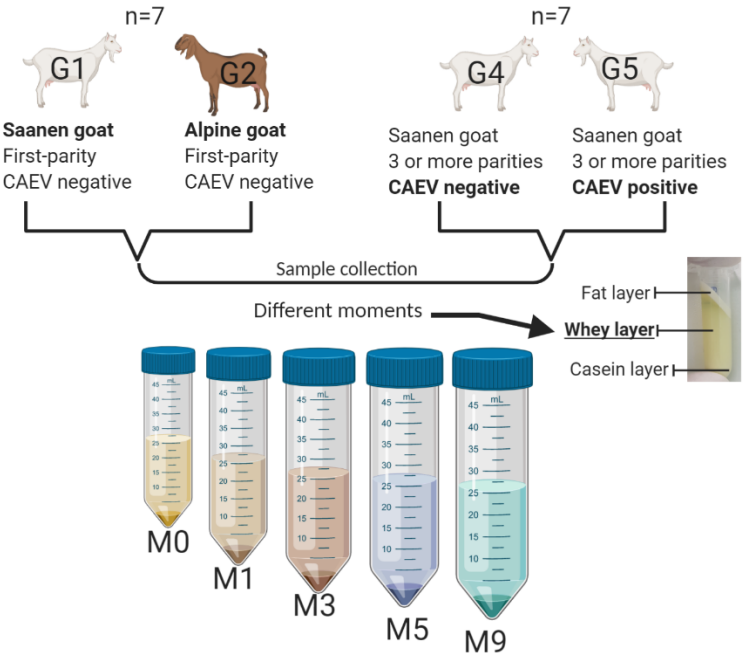
3.4.2 Samples and experimental design

To exclude the influence of diet and management effects, we collected milk of goats from the same farm, with the birth process within a short time frame. Goat milk samples were collected from 28 animals (each group with seven animals) divided into 4 groups (Figure 1) with different breeds (Saanen and Alpine) and order of parity. After the first day, all goats had somatic cell count lower than 1.0 x 10^6 cell/mL and bacterial count lower than 500.000 UFC/mL, i. e., free of

inflammatory and infection effects. Examinations were performed only to certify the health of these animals, as well as the IDGA (OIE, 2012), which was performed (2 times) to obtain assured and separate animals positive for the caprine arthritis encephalitis virus.

The animals were milked by mechanic techniques in an automatic milking system for day to day work, and samples were collected every milking from day 0 to day 9. 100 mL of milk was collected at each time point and transported for the -80°C for storage. After finishing sample collection, samples collected after 0, 1, 3, 5, and 9 days were frozen samples were transferred to the laboratory in dry ice.

Figure 1 - Organization of groups and moments used for this research with goat`s whey.



Source: Created with BioRender.com

3.4.3 Whey separation

Two mL of each homogenized sample were gathered into a pool of seven animal`s milk for processing. The samples were centrifuged at 14,000 x G/4°C for 15 minutes, six times to remove the fat layer. The middle layer which was the milk serum, was used for the Biuret assay and the 2D-PAGE electrophoresis based on results from Pozzi et al., (2015) and Cerbino et al. (2018), with the modification in the minutes and times of centrifugation.

3.4.4 Protein quantification [2]

[2] Sodium hydroxide 1,86 M, sodium and potassium tartrate320 mM, copper sulfate120 mM e potassium iodide 300 mM.

Total protein was quantified in whey using the Biuret method (Doumas et al. 1981). The protein precipitate was resolubilized in 200 µL of sodium hydroxide (500 mM NaOH). A 50 µL aliquot of the sample was transferred to a tube containing 2.5 mL of Biuret reagent that was heated in a water bath at 37 °C for 10 minutes. After five minutes of rest at room temperature, absorbance readings were taken on a spectrophotometer. An analytical curve was constructed with concentrations of 5 to 50 g L−1 from a stock standard solution of bovine albumin (100 g L−1), subsequently, their absorbances were measured at 545 nm using a spectrophotometer in the visible-UV range.

3.4.5 Electrophoretic runs [3, 4, 5]

After quantification each sample was resolubilized in 100 µl of buffer containing 7 mol L^{-1} urea (BioAgency, São Paulo, Brazil), 2 mol L^{-1} thiourea (Synth, Diadema, Brazil), 2% CHAPS (m/v) (Merck, Darmstadt, Germany), ampholytes of pH 3 to 10 at 0.5% (v/v) (Amersham Biosciences, Uppsala, Sweden); and 0.002% bromophenol blue (BioAgency, São Paulo, Brazil).

After precipitation, each sample was resolubilized in 200 µL of Tris-HCl buffer (1 M). To this buffer it was also added 1,4-dithiothreitol (DDT), in the proportion of 2.8 mg DDT for every 1 ml of buffer. With the protein concentration values previously known, dilutions were made in the same Tris-HCl buffer with DDT, so that the protein concentration of all samples was 1.5 µg $µL^{-1}$. 250 µL of the resolubilized sample and corrected for protein concentration was applied to the channels of a rehydration tray, where prefabricated tapes of 13 cm polyacrylamide gel were also placed, containing pH immobilized ampholytes with a gradient of 3 to 10.

The tapes with the respective samples were then covered with mineral oil and left at room temperature for 14 hours for complete rehydration of the tapes with the solution containing the sample. For the protein separation by isoelectric point (first dimension), the rehydrated tapes, were placed in the isoelectrofocalization (IEF) system of the EttanTM IPGphorTM3 model. The programming of electrical voltage used for separation was as follows: a) Stage 1, 500 V, 1 h and voltage accumulation 0.5 kVh; b) Stage 2, 1000 V, 1 h and voltage accumulation 0.8 kVh; c) Stage 3, 8000 V, 2.5 h and voltage accumulation 11.3 kVh; and d) Stage 4, 10000 V, 0.5 h and voltage accumulation 5.4 kVh (running condition: 20°C; 50 µA per tape; programming and conditions established by Ettan IPGphor system protocol 3).

[3] Acrilamida, N,N'-metilenobisacrilamida, tris-hidroximetil amino metano, dodecil sulfato de sódio (SDS), N,N',N,N'-tetrametilenodiamina (TEMED) e persulfato de amônio.
[4] Ácido acético 10% (v/v) e etanol 40% (v/v).
[5] Sulfato de amônio 8% (m/v), ácido fosfórico 1,6% (v/v), azul de coomassie G-250 0,08% (m/v) e metanol 25% (v/v).

After the last stage of isoelectric focusing and before the separation of proteins by molecular mass (SDS-PAGE), the tapes went through two stages of equilibrium. The first step was the reduction, in the presence of DDT, which acts in the disruption of the disulphide bridges present in the proteins; and the second stage was the alkylation, where iodoacetamide acts to prevent the reoxidation of thiol groups. For this, each tape was dipped in 5 mL of equilibration solution, where the first 1% DTT (m/v) was added and left under light agitation for 15 minutes. After this step, the tapes were again dipped in 5 mL of the same solution containing 2.5% iodoacetamide (w/v) instead of DTT and left for another 15 minutes under agitation.

Afterward, the second-dimension electrophoresis was initiated, where the tapes were placed on 15% polyacrylamide gels, previously prepared between 180x160x1.5 mm glass plates. A known pattern of proteins with a molecular weight between 14.4 and 97 kDa was applied to filter papers (8 µL) placed next to each strip, which was then covered with a 0.5% (w/v) agarose solution.

The race was performed in a SE 600 Ruby vat (Amersham Biosciences), with a voltage schedule of 100 V for 30 minutes, followed by 250 V for 3 hours and 30 minutes. The gels were then removed from the plates and immersed in a protein fixing solution for 40 minutes, and then placed in colloidal Coomassie dye solution for 48 hours, under light agitation. Three gels from each sample were made to check the equivalence or matching between the gel spots (points marked on the gel by the dye and representing one or more proteins and / or their isoforms) and the repeatability of the technique. After removing the dye with ultrapure water, the gels obtained in triplicate were scanned using a GE Healthcare scanner. Scanned images were analysed to evaluate the correlation between gel repetitions and the count of spots (protein) there in, using the Progenesis Samespot.

3.4.6 Data analysis

ANOVA was performed with Progenesis Samespot with $p<0.05$ and fold change >1.5, and for the Table construction, we used the fold change above 2.5. In this program, the data calculation assumes that the conditions are independent and applies the statistical test that assumes the means of the conditions are equal. To assess the comparison of moments between groups (G1-G2; G4-G5), the analysis is descriptive based on the highest average value analysed by the program, and descriptive for the quantification of proteins.

3.5 Results and discussion

Caprine milk, on the average, contains 4.6% fat, 3.6% protein, 2.8% lactose different from cow milk (Haenlein, Caccese, 1984; Stocco et al., 2019). It is known that significant variations occur in milk composition and yield during different seasons and stages of lactation of milking cows (Zhang et al., 2015), and the same phenomena would occur in goat milk (Park, 2017). The fat, total solids, and protein contents of the milk are high in early lactation, fall rapidly, and reach a minimum during the second to third months of lactation, and then increase towards the end of lactation (Park, 2017).

Major changes in the milk were observed between moment 0 (day of birth) and moment 3 (3 day) in Saanen group and Alpine group (Table 2) and CAEV negative goats (Table 3). In group of CAEV positive goat (Table 3), the number of proteins was decreased, and milk transition began to occur in moment 1 when compared with the day of birth (moment 0).

Table 1 - Protein content Saanen group (G1) and Alpine group (G2) in different moments after birth using Biuret assay.

Moments	Groups	
	Proteíns (µg/µL)	
	Saanen	***Alpine***
Immediately after birth (M0)	169.47	159.49
Day 1 (M1)	100.11	108.39
Day 3 (M3)	32.31	38.60
Day 5 (M5)	32.71	27.70
Day 9 (M9)	23.18	21.55

Source: Prepared by the authors

Table 2 - Protein content of the negative group (G4) for CAEV with more than 3 breeding and positive group (G5) for CAEV with more than 3 breeding in different postnatal moments using Biuret assay.

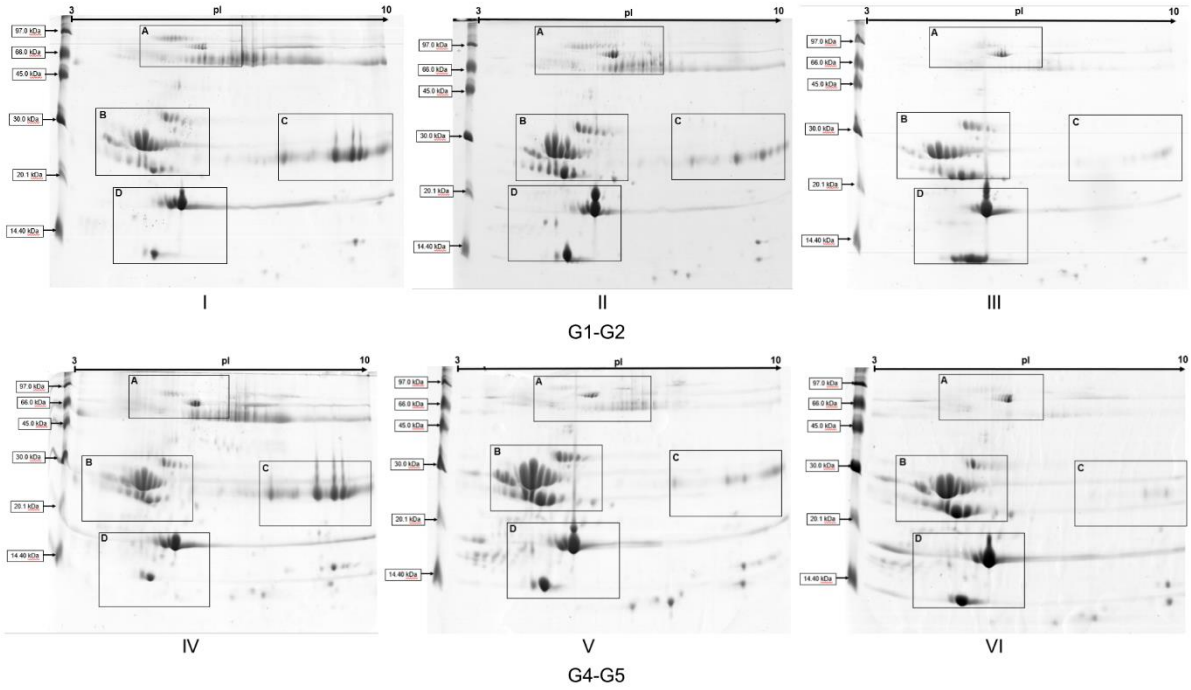
Moments	Groups	
	Proteíns (µg/µL)	
	CAEV (-)	**CAEV (+)**
Immediately after birth (M0)	87.00	59.13
Day 1 (M1)	69.17	34.85

Day 3 (M3)	32.68	34.07
Day 5 (M5)	31.67	29.43
Day 9 (M9)	14.97	24.71

Source: Prepared by the authors

A higher number of clear protein spots was observed more in the CAEV negative than for in the CAEV positive. The gels had an average number of spots ranging from 70 (comparing the breeds of goats) to 40 (comparing negative and positive goat). Thus, to consider the reproducibility of the gels with all repetitions, a linear correlation coefficient above 0.90 should be obtained, as it happened (Figure 2). The reproducibility of the gels was observed in dispersion graphs in the Progenesis Samespot, considering the percentage of the spot volume between repetitions (% vol).

Figure 2 - Gels obtained in the electrophoretic run of the goat's milk pool at different moments and groups (G1, Saanen goats; G2, Alpine goats; G1-G2: Breed comparison; G4, CAEV negative Saanen group; G5, CAEV positive Saanen group; G4-G5, Control and Infected comparison; About the moment of sample collection: I and IV, M0 (Immediately after birth); II and V, M3 (3 day); III and VI, M9 (9 day).Highlighted in the rectangles in the image are groups of proteins (A, B, C, and D). For one individual vision of this Figure redirect to the Supplementary Figure 1.



Source: Prepared by the authors

The distribution of the diversification of protein spots in the experimental groups was observed between 30.0 – 14.40 kDa and pI 4 - 10 (Figure 2), and indicated that the molecular weight (MW) and isoelectric point (pI) distributions were homogeneous among the different

experimental groups. In Figure 2, a cluster of proteins (A, B, and D) in all groups was observed in the transition period from colostrum to milk. The research made by Zhang et al. (2011) with the proteomic identification of proteins from cow milk using 2D-PAGE related with the clusters in this research. For example, cluster. A found in this research is supposed to correlate to albumin, that those authors identified using LC-MS, this protein is present in biological samples like blood and milk.

In the region B of Figure 1, proteins like casein can be identified what corroborates with the research comparing polymorphisms in Indian goat's milk protein (Kumar et al., 2013). Casein is the main protein fraction of ruminant milk, one valuable component of milk due to its nutritional value and processing properties (Zhang et al., 2011; Kumar et al., 2013). The research with bovine whey and 2D-PAGE clusters of spots with similar MW but different pI values indicate posttranslational modifications during the transition from colostrum to milk, such as phosphorylation or glycosylation (Zhang et al., 2011).

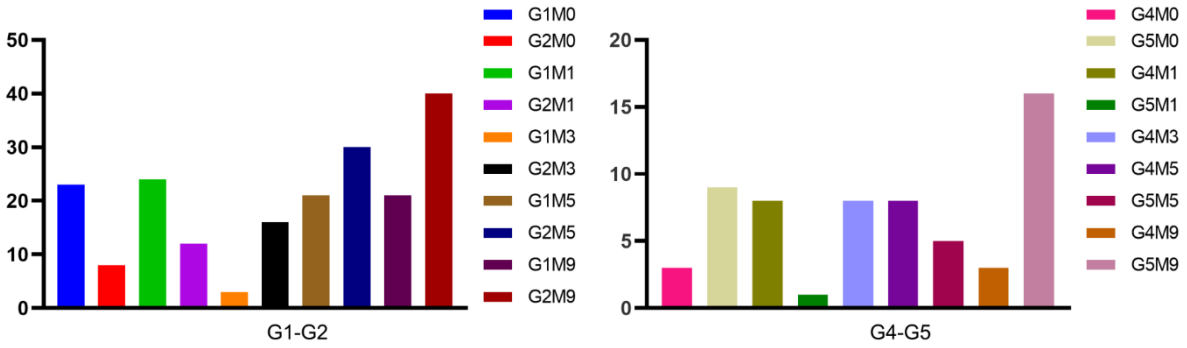
The final gestation periods it has already been reported that there is a development of the ruminant "baby" gastrointestinal system (Zabielski et al., 2002; Guilloteau et al., 2009). During the postnatal phase, the development of the digestive functions of newborn ruminants is critical in the first three days (Zabielski et al., 2002; Guilloteau et al., 2009), thus, the maintenance of protein groups in milk is an important factor over the days for adequate development of the ruminant newborn.

Some reports indicate that the pancreas and the intestines of calves at seven days of birth are not yet fully developed in fact, since the complete development of this system will happen with the introduction of solid food (Zabielski et al., 2002; Guilloteau et al., 2009). The supply of enzymes from colostrum or milk to the newborn is part of the digestion process, and these products are necessary for proper digestion during breastfeeding and for the development of a functional gastrointestinal tract (Zhang et al., 2015).

After the third day of parity, the transition period observed for the protein content is demonstrated by the changes in the key macromolecules of the sample. In a research using the transition from colostrum to milk in different moments (0, 3, 7, and 21) for bovine whey, Zhang et al. (2011) found correlated results. The authors found that the whey protein expression pattern at days 0 and 3 after calving were similar. In this study, the comparison between the breeds Saanen and Alpine (Figure 2, G1-G2) showed that the up-regulated proteins are evident in the *Saanen* samples (moments 0 and 1) and in alpine goats. The other moments had more spots, indicating an increased expression of proteins, but in general the day of birth presented more abundance in the types of spots in the gels and with 3 days the colostrum started to change for regular milk.

In this research, the authors attempted to compare two different breeds of goats, *Saanen* and *Alpine*, in the transition from colostrum to milk, but the spots in all groups are correlated with a close pI and MW. Bouniol et al. (1994) classified *Saanen* and *Alpine* like from the same country (French dairy breed) and found that the amount of αs2-Cn A, B, and C in the whey were 0.85, 0.04, and 0.11 respectively for both breeds. As the animals received the same food and shared the same environment, those results can be related to the biological aspects of the group of animals randomly chosen. *Saanen* and *alpine* goats are highly productive temperate breeds, and they are constantly facing settings beyond their comfort zones, in their native regions, as well as in foreign countries where they are imported for their attractive productive traits (Jaber et al., 2019).

Figure 3 - Spots concentration using the abundance of spots between the groups. Legend: G1, Saanen goats; G2, Alpine goats; G1-G2: Breed comparison; G4, CAEV negative Saanen group; G5, CAEV positive Saanen group; G4-G5, Control and Infected comparison. About the moments (postnatal) of sample collection: M0 (Immediately after birth); M1 (1 day), M3 (3 day), M5 (5 day) and M9 (9 day).



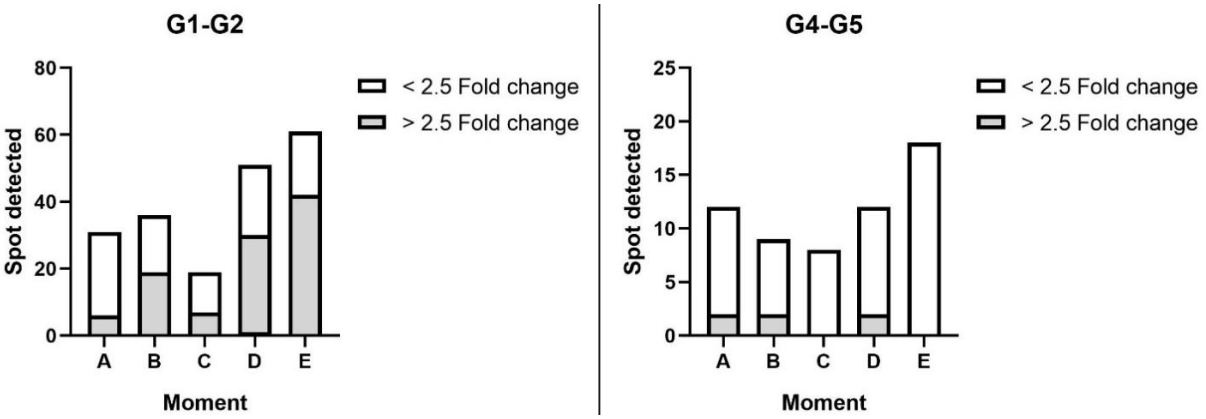
Source: Prepared by the authors

In the groups of CAEV negative goats and positive CAEV goats (Figure 3, G4-G5), the second one presents more content of spots in moments 0 and nine, although no information from literature could explain it. The seminal plasma of male goats with CAEV has been found to have unique proteins related to the virus infection as Arylsulfatase A and Clusterin, and higher sperm concentration ($P<0.05$) in seropositive males (Bezerra Júnior et al., 2017). Given the findings, the results are not influenced by the chronic infection, but by the physiological characteristics of the animals selected at random for the study. The quality of milk is negatively affected by CAEV infection with respect to total protein, fat, and lactose (Kaba et al., 2012).

These results can be the number of parity order since the animals went through more than 3 times doing the process of mammary gland milking and involution. Animals can be affected with the chronic infection as described for Blacklaws (2012) and Kaba et al. (2012), and the milk production and milk composition suffer from the resulting of virus replication in the goat

organism (Blacklaws, 2012). Animals infected with the virus can suffer with possible clinical signs and malnutrition and that can be the cause of the negative aspect in the quality of milk.

Figure 4 - Spots concentration in the group's comparison (G1-G2 and G4-G5) and different moments using the fold change. Legend: G1, Saanen goats; G2, Alpine goats; G1-G2: Breed comparison; G4, CAEV negative Saanen group; G5, CAEV positive Saanen group; G4-G5, Control and Infected comparison. About the moments (postnatal) of sample collection: A, M0 (Immediately after birth); B, M1 (1 day), C, M3 (3 day), D, M5 (5 day) and E, M9 (9 day).



Source: Prepared by the authors

Besides the milk composition, gel electrophoresis is a valid technique, even in the simplest format, especially when working with proteomes of different complexities, enriched protein fractions or sub proteomes (Jorrin-Novo et al., 2018). Two-dimensional electrophoresis can be used as a marker for the visualization of the proteome from the milk of young goats and goats with the CAE virus. With those results (Figure 4), an initial screening of large macromolecules in the different groups of animals with and without the CAE virus is presented.

3.6 Conclusion

2D-PAGE is a first step technique in proteomics which aids in the visualization of the transition period in colostrum to milk. The parity order factor was more determinant for the change in protein concentration than the fact that the animals were positive for the CAE virus. In the transition from colostrum to milk, the moment immediately after delivery was the one that showed the highest concentration of protein spots, however over time, the groups of proteins remain, but in a lower concentration. Future studies would suggest investigating the identities of proteins present and lost due to CAEV-infection in dairy farms.

3.7 Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and the writing of the manuscript.

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4 CAPÍTULO 2 - PRELIMINARY METABOLOMIC ANALYSIS OF THE TRANSITION FROM COLOSTRUM TO MILK IN GOATS WITH CAPRINE ENCEPHALITIS ARTHRITIS VIRUS (CAEV)

4.1 Resumo

Com esse estudo objetivou-se entender por meio da a metabolômica “untargeted” sobre o metaboloma do leite de cabra de animais com diferentes padrões de idade e saúde (cabras saudáveis e cronicamente positivas com o vírus da artrite encefalite caprina - CAEV), avaliando também em diferentes momentos na transição do colostro ao leite. Foram utilizadas duas comparações, animais sem o vírus da CAE de primeiro parto e animais sem o vírus com mais de três partos, a outra comparação foi desse último grupo, comparado ao grupo de animais cronicamente positivos para o vírus da CAE. Cada grupo possuía um total de 5 animais e as coletas foram realizadas em três momentos, M0 (imediatamente após o parto), M3 (com 3 dias) e M9 (com 9 dias). Para a realização das análises foi utilizado HPLC-DAD, LTQ-Velos, com analisadores Orbitrap para baixa e alta resolução, para limpeza dos dados o programa MzMine e para análises estatísticas o programa MetaboAnalist. Um total de 73 metabólitos foram identificados por meio de LC-MS. Os metabólitos identificados pertencem principalmente as classes dos lipídios, no caso ácidos graxos, seguidos de peptídeos, aminoácidos, carboidratos outros compostos orgânicos. A concentração total de metabólitos mostrou-se distinta entre os grupos experimentais e na transição do colostro para o leite das cabras. Nos animais jovens saudáveis no M0 (após o nascimento), houve uma maior concentração de metabólitos. Quando comparados os dados dos animais negativos e positivos para CAEV, não foi observada diferença ($P>0.05$). Dentre o grupo de metabólitos encontrados, estão os ácidos graxos (ácido oléico, ácido palmítico, entre outros), açucares (sucrose, D-maltose, entre outros), além de outros compostos orgânicos (riboflavina). Os metabolitos observados fornecem uma visão abrangente das diferentes amostras de leite de cabra, e confirmam alterações do metaboloma durante a transição do colostro para o leite de cabras, assim como entre animais mais jovens comparado com animais que já passaram por vários processos de lactação. Com esse estudo, buscou-se mais informações sobre o perfil metabolômico do leite de cabra, confluindo com uma direta contribuição em estudos ômicos com leite de ruminantes e para produção animal.

Palavras-chave: biotecnologia. ruminante. metabólitos. espectrometria de massa.

4.2 Abstract

With this research the aim is to understand through the "untargeted" metabolomics about the goat milk metabolome of animals with different age and health standards (healthy and chronically positive goats with the caprine arthritis encephalitis virus - CAEV), also evaluating moments in the transition from colostrum to milk. Two comparisons were used, animals without the CAE virus from the first parity and animals without the virus with more than three orders of parities. Another comparison was from the following group, compared to the group of chronically positive animals for the CAE virus. Each group had a total of 5 animals and the sample collections were performed in three moments, M0 (immediately after delivery), M3 (with 3 days), and M9 (with 9 days). For the analysis, HPLC-DAD, LTQ-Velos, with low and high-resolution Orbitrap analyzers were used. To clean the data was used MzMine program and for statistical analysis the MetaboAnalist program. A total of 73 metabolites were identified using LC-MS. The identified metabolites belong mainly to the lipid classes, in this case, fatty acids, followed by peptides, amino acids, carbohydrates, and other organic compounds. The total concentration of metabolites was different between the experimental groups and in the transition from colostrum to goat's milk. In healthy young animals at M0 (after birth), there was a higher concentration of metabolites. When comparing the data of the negative and positive animals for CAEV, no difference was observed (P> 0.05). Among the group of metabolites found are fatty acids (oleic acid, palmitic acid, among others), sugars (sucrose, D-maltose, among others), in addition to other organic compounds (riboflavin). The observed metabolites provide a comprehensive view of the different goat's milk samples and confirm changes in the metabolome during the transition from colostrum to goat's milk, as well as among younger animals compared to animals that have already supported various lactation processes. In this study was produced more information on the metabolomic profile of goat's milk, converging with a direct contribution in omic studies with ruminant milk and for animal production.

Keywords: biotechnology. ruminant. metabolites. mass spectrometry.

4.3 Introduction [6]

Milk offers the best physiological nourishment to the neonate, with its composition used to estimate the nutritional requirements of infants and to guide the composition of infant formulae (Scano et al., 2016). In the case of ruminants, the respective species works like human milk, the newborn from goat and cow show better nutrition aspects from his mother colostrum/milk. As a consensus, milk is one of the most important resources of nutrition for newborns (Zhang et al., 2011; Zhang et al., 2015). Milk has higher content of nutrients, they are consisting of water, lipids, carbohydrates, proteins, vitamins, minerals, and a diversity of small secondary metabolites (Rocchetti et al., 2020) is important for the development of the neonate ruminant.

The “placenta” of ruminants is known as syndesmocorial type (sinepithelium corial), which characterizes a certain degree of difficulty in transplacental passage of molecules such as immunoglobulins (Santos et al., 2017). Colostrum’s importance in the formation of essential immunity to neonates (Santos et al., 2017). Colostrum promotes protection to the immune system of newborns and assists in passive immunity against certain pathogens (Sanchez-Macías et al., 2014).

Considering the gastro-intestinal tract (GIT) the absorption of nutrients in milk-fed ruminants is important, for example, fatty acids and glucose is the source of energy and triggering the development of the immature tissue (Baldwin et al., 2004).The consumption of colostrum and milk is essential for the newborn because the GIT development requires nutrients in milk and those affects feed intake, the efficiency of digestion, and resistance to gastrointestinal disorders, and thus animal growth and health, each method enhancing these processes are highly desirable (Górka et al., 2018) reflecting in the overall growth of the ruminant. With that, the type and quality of the colostrum/milk have importance.

Therefore, although animal genetics strongly influence the lipidic profile of milk, the main factors are related to dairy cow nutrition (Rocchetti et al., 2020). Other factors that can influence the metabolomic aspect of food are diseases. One viral disease can affect goats and sheep is the Caprine arthritis-encephalitis (CAE), which is a chronic and progressive infectious disease of goats, a counterpart of Maedi-Visna disease in sheep, both caused by an infection with a retrovirus belonging to the genus (SRLV) of small ruminant lentivirus (Milczarek et al., 2019). Because some virus infections may lay dormant for extended periods, the animals may also remain asymptomatic until later progression results in apparent arthritis and weight loss (Leitner et al., 2010; Milczarek et al., 2019).

[6] Food Research International - ANEXO 3

The identification of several metabolites whose concentration differed between infected and uninfected goats, and different age of production too (Milczarek et al., 2019). Milczarek et al. (2019) discusses the identity and quantification of metabolites in adult dairy goats not only can suit the needs of veterinary and production studies but also for those human medicine studies which employ goats as models of human diseases (Milczarek et al., 2019).

To investigate the nutrition and infectious diseases reflect of that in the milk, some tools are necessary, metabolomic analysis represents a novel tool that could easily be translated into practical use with the major advantage that the sample can be obtained in a non-invasive manner. Overall, this is one of the first consisting of a comprehensive metabolomics-based analysis and the aim is to investigate possible correlations between metabolites composition from goats in different parity order, and animals chronic infected with caprine arthritis-encephalitis virus.

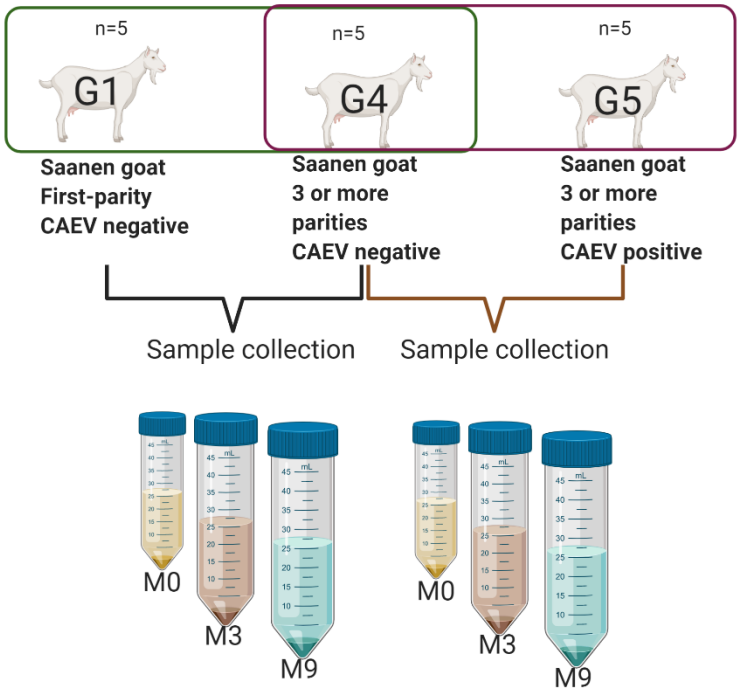
4.4 Material and methods

4.4.1 Goats and milk collection

The study was carried out through a partnership a farm located in São José do Rio Preto/SP (20°53'26.5"S 49°20'41.8"W) with the translational medicine from the UNIFESP and the analytical department from ICQ-USP with the direction of the animal ethics and experimentation committee (CEUA) and approved with the following code - FOA, number 00422-2018.

For this research, was used 15 goats (5 in each group) with different production age, health standards, and times of lactation. The animals were natural and chronic infected with SRLV over years, since the positive animals stay in the same place at the farm, as shown by regular serological monitoring using ELISA (Kaba et al., 2011). The animals had the microbiology and SCC (somatic cell count) evaluated following heathy requirements for the samples (Zhang et al., 2015). Just animals without any other alteration were picked for this research. The animals with SRLV could not have other associated diseases since the focus is to understand the aspect of the virus in milk and mammary gland.

Figure 1 - Representative image of the organization of the groups of animals studied.



Source: Created with Biorender.com

The collection (Figure 1) of the milk happens in tree moments (day of birth, with 3 days and 9 days), following Zhang et al (2015) with modifications. The whole milk and the blood serum for the CAE virus ELISA were frozen and stored at −20 °C for serological testing (all samples) and at −80 °C for metabolomic analyses. In the laboratory, the whey was separated by centrifuging and harvested into aliquots.

Table 1 - Experimental groups and the moments used for the research about the transition from colostrum to milk in goats.

Groups	General information
Saanen group (G1)	Afterbirth moments of sample collection: M0 (Immediately after birth), M1 (1 day), M3 (3 days), M5 (5 days) and M9 (9 days)
Saanen CAEV negative (G4)	**
Saanen CAEV positive (G5)	**

Source: Prepared by the authors; ** Same organization of sample collection from the first line

4.4.2 Preparation of the samples

Samples stored at −80°C were thawed on ice, underwent a stirring process for 2 minutes, then aliquots of 100 µL were made. In each aliquot, 300 µL of MeOH / EtOH (1: 1) was added, after this process the samples were stirred for 1 minute and centrifuged (20 min, 13000 rpm, 15ºC). The supernatant was removed and filtered, using a 0.2 µm filter, the filtered material had

the addition of 10 µl of P.I (Methionine sulfone) and was stored again at −20 ° C until the moment of analysis.

QC (quality control) was prepared from the 10 µl aliquot of each milk sample, from which it was inserted in a single vial and a pool was prepared. Subsequently, this pool (QC) was stirred and the extraction process was carried out, according to the protocol described above. The blank was prepared from a plier of 100 ul of ultrapure water; then an extraction solution was added, and the same processes described above were carried out. As for the preparation of the Internal Standard (P.I), methionine sulfone (1mg / ml) was used, which was prepared with the addition of acetonitrile (ACN) and ultrapure water (40:60), these products were stirred and sonicated for 10 minutes.

4.4.3 Metabolomic analysis of milk

The HPLC-DAD hyphenated mass spectrometer consisted of an LTQ-Velos, with ion-trap and orbitrap analyzers for low and high resolution. The reagents used were methanol (MeOH), ethanol (EtOH), acetonitrile (ACN) obtained from Fluka Analytical, Germany; 98% formic acid obtained from Sigma Aldrich, Germany; ultrapure water (Milli-Q-Utra Pure System (Millipore-USA). All solvents were grade LC-MS. The source used was a HESI-II, at a temperature of 250 ° C and the voltage applied to the 4 kV source. 35 and 10 µ.a of the sheath and auxiliary gases (N2) were used. During the analysis, the samples were kept refrigerated at 10 ° C on the rack of the automatic injector. The chromatographic column used was C18. The column was maintained at room temperature, controlled at 22 ° C.

The analysis mode in the mass spectrometer was carried out with high-resolution acquisition (Orbitrap), in the positive and negative full-scan mode, analyzing from 50-1200 m / z. For DAD, an analysis of 220-600 nm and 20 Hz of data acquisition frequency was used. The tension applied in the MS source was 3.5 kV, and the column used was the Ascentis Express 10cm x 2.1mm with 2 um particles with a flow of 300 ul/min. The chromatographic method consisted of a gradient with Methanol (0.1% formic acid) as phase B and ultrapure water (0.1% formic acid) as phase A. The gradient started with 5% phase B for 5 min, then going to 100% B for up to 50 min. From 50 to 58 min the phase remained at 100% B, returning in 59 min to 5% B, up to 65 min. The flow was constant during the 300 µL/min run.

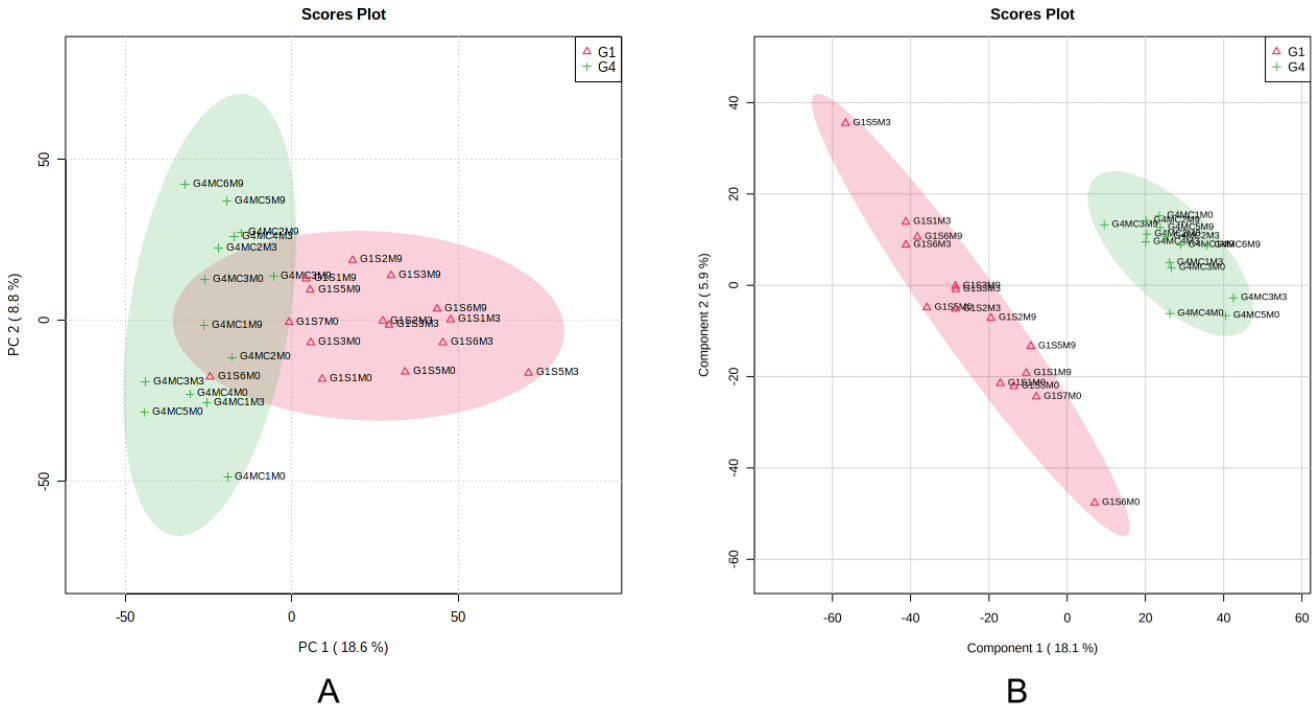
4.4.4 Statistical analysis

The MzMine was used for raw peaks extraction, data baselines filtering and calibration, peak alignment, deconvolution analysis. All comparisons were imported into the online analysis platform Metaboanalyst 4.0 (http://www. metaboanalyst.ca/). Next, data was performed a multivariate analysis, including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA). PCA was used to visualize the data set and display the similarity and difference. The PLS-DA model was validated by fold-change permutation tests to check its validity. Statistical significance was defined at $p<0.05$, with highly significant values at $p < 0.01$. To refine this analysis, the variable importance for the projection (VIP) values along the predictive component were obtained. Using mummichog (Li et al., 2013) the metabolites ($p<0.05$) were further identified and validated by searching the online databases including the Kyoto Encyclopedia of Genes and Genomes (KEGG), Livestock Metabolome Database (LMDB). With mummichog (Li et al., 2013) each differential metabolite was then cross listed with the pathways in the KEGG, and the top altered pathways were identified and finally constructed according to the potential functional analysis.

4.5 Results and discussion

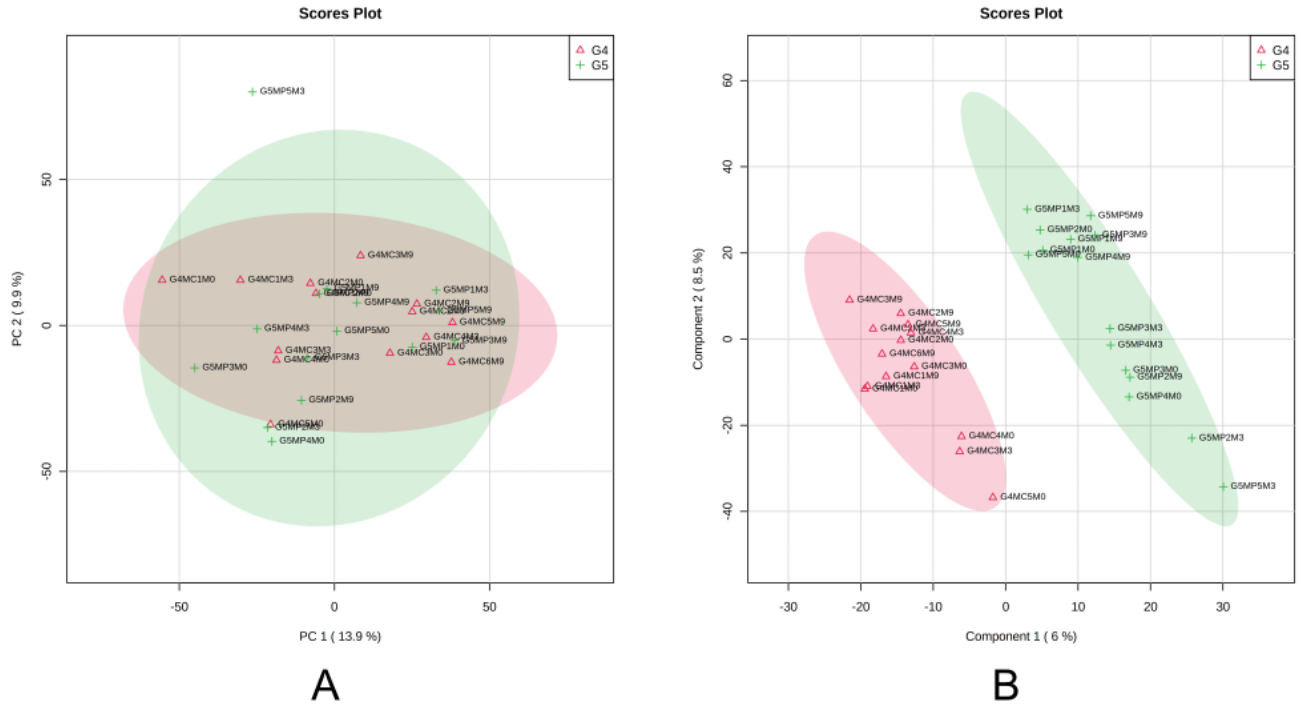
The PCA score plot showed a clear separation between the first birth group (G1) and the group with more than three births (G4), (Figure 1 and Supplementary Figure 2-5), but in the group with CAEV negative goats (G4) and positive goats (G5) we didn`t find statistic difference (Figure 2 and Supplementary Figure 6-8). These results suggest that numbers of lactation can influence in the metabolites profile.

Figure 2 - Principal component analysis (PCA) score map and partial least squares discriminant analysis (PLS-DA) score map of the whey metabolites from 10 lactating goats in different conditions. (A) PCA scores plot from explicit components between the first birth group (G1) and the group with more than three births (G4), the explained variances are shown in brackets. (B) PLS-DA scores plot from explicit components between the groups. All numbers in parentheses are the percentage of explained variation of goat milk metabolites profiles and the groups nominated above.



Source: Prepared by the authors

Figure 3 - Principal component analysis (PCA) score map and 2-D partial least squares discriminant analysis (PLS-DA) score map of the whey metabolites from 10 lactating goats in different conditions. (A) PCA scores plot from explicit components between CAEV negative goats (G4, n=5) and CAEV positive goats (G5, n=5), the explained variances are shown in brackets. (B) PLS-DA scores plot from explicit components between the groups. All numbers in parentheses are the percentage of explained variation of goat milk metabolites profiles and the groups nominated above.



Source: Prepared by the authors

Using PCA, a clear separation of the transition from colostrum to milk happens in the first parity animals in the comparison 3 days versus 9 days (Supplementary Figure 9). The group with more than 3 orders of parity negative for CAEV (G4) in comparison with the CAEV positive group (G5), the PCA does not show a clear separation (P>0.05).

According Yang et al. (2016) and O'Callaghan et al. (2018) as basic health conditions of the udder, the food provided to the animals, the ruminal metabolism, the parity level and lactation stage are directly related with the functioning of the breast tissue, as well as the basic health conditions of the animals. Those aspects are related to goats' milk since they are small ruminants with evolutive similarities with bovines. The VIP score of metabolites in those moments (M0, M3, and M9) was above 11, the accepted value is above 1 (Mendez et al., 2020), represents the difference between metabolites in those different groups and the features can show one reliable identification.

The averaged unsupervised hierarchical cluster analysis (HCA) produced from the foldchange-based heat map resulted in two main groups of variation as we can see with the

difference of colors (Supplementary Figures 4 and 7). With the first group of parity (G1) compared with the CAEV negative group (G4) the cluster hierarchically represented the metabolites profile of those samples, and with that, the results show more concentration of metabolites in the young animals considering the different moments' technical replicates (Supplementary Figure 4).

But when the comparison is between the CAEV negative animals (G4) and CAEV positive animals (G5), the variation of features presented a low chemical similarity between the groups. The heat map clearly showed that comparison with G1 and G4 samples possessed a cluster including less abundant compounds when compared, showing clear aspects related to the literature since more old animals present alterations in the quality of milk. The results in the Supplementary Figure 7, we can speculate that milk was lacking some components, not because CAEV infection, but that can be related with evolution aspects of the animals, with those results lack a piece of direct information about the influence of the virus in the metabolites features in the transition from colostrum to milk in goats.

Overall, more than 5000 molecular features (possessing different composite mass spectra) obtained across samples, and that can be correlated to 1226 possible structures (i.e., metabolite species). With all the methods and the general analysis of the data, was identified 73 unique structures. Foroutan et al. (2019), explain because of the complexity of the food matrix under investigation a wide number of chemical features can be obtained by using untargeted metabolomics (Foroutan et al., 2019).

Table 2 - Organic acids and Carbohydrate identification by KEGG present goats milk using mummichog (p<0.05).

Class	Metabolite name	Query mass	Matched compound	ID
Organic acids	Oleic acid	283.2626	C00712	01
	L-Lactic acid	91.03857423	C00256	02
	Hydroxypropionic acid	91.03857423	C01013	03
	Pyroglutamic acid	130.0494292	C01879	04
	Pyrroline hydroxycarboxylic acid	130.0494292	C04281	05
	Hippuric acid	180.0650981	C01586	06
	Gamma-Linolenic acid	279.2312846	C06426	07
	Alpha-Linolenic acid	279.2312846	C06427	08
	Palmitic acid	257.2469465	C00249	09
	Oxoadipic acid	161.0439196	C00322	10
	2-Aminobenzoic acid	138.0543633	C00108	11
	2-Methyl-3-oxopropanoic acid	103.0385844	C00349	12
	2-Ketobutyric acid	103.0385844	C00109	13
	Acetoacetic acid	103.0385844	C00164	14
	Stearic acid	285.2781836	C01530	15
	Oxoglutaric acid	147.0284141	C00026	16

	(GlcN)1 (Ino(acyl)-P)1 (Man)1	342.307808	G00146	17
	27-Deoxy-5b-cyprinol	437.3620559	C05446	18
	Glycerylphosphorylethanolamine	216.0624933	C01233	19
	Phenylacetylglycine	194.0806721	C05598	20
	Malonyl-Carnitin	162.1119804	C00487	21
	Succinic acid semialdehyde	103.0385844	C00232	22
	Glycerophosphocholine	258.10943	C00670	23
	Ketoleucine	131.0699328	C00233	24
	Sphingosine	300.2892104	C00319	25
Carbohydrates	Sucrose	343.1228006	C00089	26
	Melibiose	343.1228006	C05402	27
	Isomaltose	343.1228006	C05400	28
	Beta-Cortol	343.1228006	C01235	29
	Alpha-Lactose	343.1228006	C00243	30
	1-beta-D-Glucopyranosyl-4-D-glucopyranose	343.1228006	C00185	31
	Trehalose	343.1228006	C01083	32
	D-Maltose	343.1228006	C00208	33
	Dihydroxyacetone	91.03857423	C00184	34
	D-Glyceraldehyde	91.03857423	C00577	35

Source: Prepared by the authors

The entire list of goat's milk metabolites annotated according to this experiment workflow is presented in Tables 2-4 and the interaction between some of the compounds is in Supplementary Figure 1. The list of compounds found is composed mainly of lipids like fatty acids and triglycerides, peptides and amino acids, and some of them are related to the steroid's groups. In one research with cow milk-related with different types of feeding regimens, the authors find correlated groups of metabolites (Rocchetti et al., 2020). To summarize, the compounds confirmed by LC-MS (Tables 2-4) some have been classified mainly in fatty acids (FA) like oleic acid (Table 2), palmitic acid, and stearic acid, as cited by the literature.

Those are the main fatty acids components in the fat layer of goat milk (Alonso et al., 1999; Lad et al., 2017), and they present important nutritional aspects for the development of the newborn. Using FA in neonate rats, a positive influence in gastrointestinal microbial ecology, and that will favor activity, positively influencing the biological aspects of these animals and highlighting the aspect of goat's milk as a functional food (Lad et al, 2017; Lopez et al., 2019).In the young growing ruminant, variations in the type and form of nutrients supplied to the gastrointestinal tract may alter cellular proliferation, total nutrient use by the gut, and ultimately, nutrients available to support growth (Baldwin et al., 2004).

Colostrum intake has a systematic effect on the metabolism and endocrine status of neonates, for example, a higher amount of fat in this milk will contribute to more ingested fat and increased fat absorption improving the energy status in colostrum-fed calves (Hammon and

Blum, 1997). Hammon and Blum (1997) explain the energy intake by sugars in this component and with that the increase of enzymes like lactase in the small intestine, and the gluconeogenesis development in the organism. In Table 2, it`s the identification of carbohydrates like sucrose, alpha-lactose, D-maltose, which can influence the energy aspect for the newborn during the colostrum/milk consumption during the first days of life. Important aspects of the development of the neonates, because, increasing energy, other related aspects can increase the survival of these young animals in the first days after birth.

Table 3 - Amino acids and Nucleotides identification by KEGG present goats milk using mummichog (p<0.05).

Class	Metabolite name	Query mass	Matched compound	ID
Amino acids	5-Aminoimidazole ribonucleotide	296.0653095	C03373	36
	Betaine	118.0858999	C00719	37
	L-Valine	118.0858999	C00183	38
	Glycine	76.03950025	C00037	39
	L-Leucine	132.1015144	C00123	40
	L-Isoleucine	132.1015144	C00407	41
	L-Arginine	175.1183826	C00062	42
	D-Arginine	175.1183826	C00792	43
	L-Proline	116.0702226	C00148	44
	D-Proline	116.0702226	C00763	45
	Beta-Alanine	90.0546866	C00099	46
	L-Alanine	90.0546866	C00041	47
	Sarcosine	90.0546866	C00213	48
	D-Glutamine	147.0759578	C00819	49
	L-Glutamine	147.0759578	C00064	50
	Phosphocreatine	212.0423325	C02305	51
Nucleotides	2-Dehydro-3-deoxy-L-fuconate	163.0595879	C03827	52
	Adenosine monophosphate	348.0709401	C00020	53
	2'-Deoxyguanosine 5'-monophosphate	348.0709401	C00362	54
	Uridine	245.076487	C00299	55
	Cyclic AMP	330.0596214	C00575	56
	Guanine	152.0573162	C00242	57

Source: Prepared by the authors

Table 4 - Organic compounds and vitamin identification by KEGG present goats milk using mummichog (p<0.05).

Class	Metabolite name	Query mass	Matched compound	ID
Organic Compounds	3-Dehydrosphinganine	300.2892104	C02934	58
	4-Hydroxy-5-phenyltetrahydro-1,3-oxazin-2-one	194.0806721	C16595	59
	3-Carbamoyl-2-phenylpropionaldehyde	194.0806721	C16587	60
	(R)-3-Ureidoisobutyrate	147.0759578	C21029	61
	(S)-Methylmalonic acid semialdehyde	103.0385844	C06002	62
	Aminoacetone	74.0597215	C01888	63
	3-Aminopropionaldehyde	74.0597215	C05665	64
	4,6-Dihydroxyquinoline	162.0545982	C05639	65
	Quinoline-4,8-diol	162.0545982	C05637	66
	4a-Hydroxytetrahydrobiopterin	258.1197913	C15522	67
	1-Nitronaphthalene-5,6-oxide	190.0493641	C14800	68
	1-Nitronaphthalene-7,8-oxide	190.0493641	C14802	69
	4-Aminobutyraldehyde	88.07527706	C00555	70
	1-Pyrroline-4-hydroxy-2-carboxylate	130.0494292	C04282	71
	Citalopram aldehyde	296.1075858	C16612	72
Vitamin	Riboflavin	377.1449609	C00255	73

Source: Prepared by the authors

For the development of neonates, other metabolites in our results can present a crucial part, for example, the sphingosine is one, that metabolite is cited as the backbone for sphingolipids construction. The first component positively influences cell development, growth, angiogenesis, and skin protection against bacterial colonization, as can protect against inflammation and the sphingolipids perform structural functions in cells and are related with cell signaling and differentiation (Miazek et al., 2016; Norris et al., 2019). The ingestion of metabolite as sphingosine it may be linked to the factors that lead to the development of the neonate, since this component, in addition to stimulating cell development, still has antimicrobial potential, since the first days of postnatal life are critical.

A diverse profile was obtained, as is possible to see with the identification of citalopram aldehyde in Table 4, this is a precursor of citalopram, important medical product for depression treatment (Rochat et al., 1999). This information can lead to more research about this component in goat milk, bringing more industrial power for this product. Other interesting products are amino

acids as valine and leucine, which are correlated with the fatty acids' composition (Kenney et al., 1962), bringing physical characteristics to goats' milk.

The data was processed and considered as potential target pathways listed in Supplementary Table 1. In the goat milk data, is possible observe the biosynthesis of unsaturated fatty acids and glycerophospholipid metabolism pathways. One research comparing different species explain the glycerophospholipid metabolism pathway is conserved in milk metabolome of ruminants and the biosynthesis of unsaturated fatty acids was shared in the non-ruminant animals as camel and horse (Yang et al., 2016). Those same authors explain those pathways can be related to the rumen metabolization and then the transfection between blood and milk production of metabolites (Yang et al., 2016).

Understanding those aspects of metabolomics of goat's milk, it`s crucial for bringing more information and more interest in the research and industrial field. Milk it`s one of the most body fluids used in metabolomics research, after plasma, but in one systematic review published in 2017, Goldansaz et al. (2017) pointed until the time of the construction of the paper, exists just a few publications with metabolomics from goats milk samples, and just one paper publish with colostrum, so this is a gap with the use of technology as metabolomics for goat milk. Goldansaz et al. (2017) summarize species like goats, sheep, and pigs are less frequently studied livestock species for the "omics". With this data, the scientific field will have recent information about goat`s milk metabolome, and metabolites related to the development of the newborn ruminant, besides that, one of the goals of this research is to change the scenario about publishing with metabolomics of goats milk.

4.6 Conclusion

A higher concentration of metabolites was observed in the milk of younger animals. Animals with and without the CAE virus had no difference in the expression of metabolites. When analyzing the transition from colostrum to milk, the moment immediately after delivery had a higher number of metabolites, compared to the other moments. Among the groups of identified metabolites, fatty acids, sugars, amino acids and other organic compounds were found. This study then contributes as one of the few studies carried out with metabolomics of goat's milk, as well as present information on the milk of chronically positive animals for the CAE virus.

4.7 Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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5 CAPÍTULO 3 - AN *IN SILICO*, STRUCTURAL, FUNCTIONAL, AND BIOLOGICAL ANALYSIS OF LACTOFERRIN OF-DIFFERENT MAMMALS

5.1 Resumo

O leite contém importante papel na nutrição animal e é constituído por diversos compostos como a proteína lactoferrina que apresenta um perfil antimicrobiano e antioxidante. Devido a importância dessa proteína o presente estudo objetivou avaliar *in silico* as propriedades químicas, biológicas e estruturais da lactoferrina em diferentes espécies de mamíferos.Para isso, utilizou-se o UNIPROT e o GenBank para obtenção da sequência de aminoácidos e confirmação dessa sequência de acordo com genoma dos animais. A modelagem foi realizada utilizando os programas: I-TASSER, Expasy ProtParam, SWISS-MODEL, MEGAx, ente outros. A arquitetura estrutural geral da proteína das 20 espécies de mamíferos demonstrou-se conservada. No estudo filogenético e no padrão da concentração de aminoácidos, as espécies que compartilham similaridades genéticas pemaneceram no mesmo cluster, assim como a concentração de aminoácidos também, havendo apenas alguns desvios. De acordo com os dados químicos e de estrutura primária e secundária da proteína, a lactoferrina apresenta termoestabilidade, assim como a quantidade de alfa Helix é maior do que as outras partes da estrutura da proteína, confluindo numa melhor estabilidade. Os dois principais desvios estruturais encontrados foram nos modelos obtidos com dados da *Capra hircus* e *Macaca mulatta*. Estes dois desviam estruturalmente das outras espécies, sendo essas inserções de 46 (posição 406-450) e 37 (posição 295-331) aminoácidos, respectivamente. No que diz respeito aos locais de ligação do ferro dentro da lactoferrina, todos os modelos têm o mesmo sítio de ligação do ferro que é característico da superfamília das transferrinas. Os programas utilizados foram capazes de gerar modelos confiáveis da lactoferrina. Esses achados podem ser usados em estudos futuros para avaliar a abordagem da biologia estrutural e o possível perfil medicinal do leite e suas proteínas, aliados à necessidade do uso de proteínas como a lactoferrina.

Palavras-chave: Estrutura 3D. Protetivo. Evolução. Modelagem.

5.2 Abstract

Milk plays a role in animal nutrition and consists of several compounds as lactoferrin protein that has an antimicrobial and antioxidant profile. Due to the importance of this protein, the present study aimed to evaluate *in silico* the chemical, biological and structural properties of lactoferrin in different species of mammals. For this, UNIPROT and GenBank were used to obtain the amino acid sequence and confirm this sequence accordingly with the animal genome. The modeling was performed using the programs: I-TASSER, Expasy ProtParam, SWISS-MODEL, MEGAx, among others. The general structural architecture of the protein of the 20 mammalian species proved to be conserved. In the phylogenetic study and the pattern of the concentration of amino acids, species that share genetic similarities remained in the same cluster, as well as the concentration of amino acids as well, with only a few deviations. According to the chemical and primary and secondary structure data of the protein, lactoferrin has thermostability, as well as the amount of alpha Helix is greater than the other parts of the protein structure, converging into better stability. The two structural deviations found were in the models obtained with data from *Capra hircus and Macaca mulatta*. These two deviates structurally from the other species, these inserts being 46 (position 406-450) and 37 (position 295-331) amino acids, respectively. Regarding the iron-binding sites within lactoferrin, all models have the same iron-binding site that is characteristic of the transferrin superfamily. The programs used were able to generate reliable models of lactoferrin. These findings can be used in future studies to evaluate the approach of structural biology and the possible medicinal profile of milk and its proteins, together with the need to use proteins such as lactoferrin.

Keywords: 3D structure. Protective. Evolution. Modelling.

5.3 Introduction [7]

Lactotransferrin or lactoferrin (LTF) is an iron-binding glycoprotein of about 80 kDa, a member of the transferrin family, and present in the colostrum and milk of mammals (Niaz et al., 2019; Liu et al., 2020). The LTF is a pleiotropic functional nutrient that can display multiple physiological actions and also is involved in different pathologies (Mayeur et al., 2016). This protein is one of the most abundant proteins in human and bovine milk, previously described as having many health benefits (antimicrobial, anti-inflammatory, iron binding etc.) and is part of different products of human nutrition (Navarro et al., 2018).

Lactoferrin has been shown to exert antibacterial, antifungal (Fernandes, Carter, 2017) and antiviral activities (Pietrantoni et al., 2003; Bruni et al., 2016), contributing to the lower prevalence and duration of infection in breast-fed infants (Breakey et al., 2015). In addition, LTF has been shown to prevent biofilm formation, due to the ability of inhibiting microbes from adhering, colonizing and forming biofilm on host cells, which is a crucial step in the development and persistence of bacterial infection (Lönnerdal, 2009).

Associated with LTF, transferrin (TF) is one iron-binding protein belonging to the superfamily distributed and strongly present in vertebrates' evolution, which is divided into serum transferrin, melanotransferrin, lactotransferrin and ovotransferrin. LTF is thought to have arose due to a gene duplication event, leading to three copies of TF family members present in mammals (Lambert, 2011).

TF main function is cellular iron transport; melanotransferrin function is not completely understood to date but it is known to be involved in melanoma cell proliferation (Dunn et al., 2006). LTF whose role is widely accepted as being part of the innate immune response. Serum TF is commonly synthesized in the liver and secreted into the plasma, is possible find mRNA synthesized in other tissues in the animal body (Asmamaw, 2016).

One of the places for storage of LTF are blood cells, in the granules of neutrophils, which strongly indicates that it plays an important role as a host defense protein (Vogel, 2012). It is mainly present in fluids since it is found in most mucosal secretions such as saliva, tears, bile, pancreatic juice, intestinal mucus, seminal fluid, and genital secretions (Alexander et al., 2012). The synthesis and secretion of LF by the exocrine glands are constant (Mayeur et al., 2016).

The LTF is present in various biofluids, such as tears, saliva, as well as nasal and genital secretions, where it could help protect us from invading bacteria (Vogel, 2012). LTF can work protecting the organism from Reactive oxygen species (ROS), which result in mitochondrial

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dysfunction and genomic damage during the aging development (Zheng et al., 2020). Experiments using mice show almost 57% reduced aging damages when the 16 weeks old mice receive LTF (Mulder et al., 2018; Zheng et al., 2020). LTF also demonstrate neuroprotective role in neurodegenerative disorders, as a therapeutic candidate for the treatment of Alzheimer's disease (Guo et al., 2017).

The information correlated with proteins and nucleotides are involved in biological processes all over the mammalians organisms, and some variety of *in vitro* and *in vivo* experimental methods have been applied in the past decade generating one large amount of data (Zang et al., 2019). Omics and bioinformatic tools will provide the biotechnological knowledge for the cost-effective production and food engineering–related commercialization of functional foods containing bioactive peptides (Carrasco-Castilla et al., 2012).

For understanding more from LTF *in silico* approaches can be used. Homology modelling of structurally unknown proteins plays an ever-increasing role in functional understanding of proteins and in drug discovery (Carrasco-Castilla et al., 2012). The present study uses *in silico* techniques and technologies to model the structures primary, secondary and tertiary, as well as chemical, binding sites and biological aspects, from the amino acid sequence of lactoferrin from 20 species of mammals.

5.4 Materials and methods

5.4.1 Sequence recovery and verification

In this study was used amino acids and nucleotides sequences of LTF from 20 mammalians from different species, including one sequence of this hybrid originated from a cross between species of *Bos Taurus* and *Bos indicus*, named of Hybrid TB (Table 1). The databases used were was the UNIPROT (https://www.uniprot.org/) for proteins and the GenBank for nucleotides sequences, following for the Ensembl eukaryotic genome annotation Project (https://www.ensembl.org/). The quality of all the nucleotides and sequences were verified using software MEGAX (Thompson; Higgins; Gibson, 1994) and the Expasy translate tool (https://web.expasy.org/translate/), and Protein Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for the protein's verification.

Table 1 - Protein and Nucleotides code of the 20 species of mammals.

Number	Species	Uniprot (protein code)	Nucleotides code
1	*Mus musculus*	P08071	ENSMUST00000035077
2	*Bos taurus*	P24627	ENSBTAT00000001704
3	*Capra hircus*	A0A452EY47	ENSCHIT00000024805
4	*Equus caballus*	A0A3Q2HKG3	ENSECAT00000033748
5	*Sus scrofa*	Q6YT39	ENSSSCT00000030564
6	*Pongo abelli*	H2PAY1	ENSPPYT00000016208
7	*Pan paniscus*	A0A161IL64	ENSPPAT00000022268
8	*Macaca mulatta*	F7BYZ5	ENSMMUT00000020008
9	*Rattus novergicus*	D3ZAB1	ENSRNOT00000047752
10	*Oryctolagus cuniculus*	G1TFW8	ENSOCUT00000029304
11	*Ailuropoda melanoleuca*	G1M1B2	ENSAMET00000013673
12	*Loxodonta africana*	G3T0S5	ENSLAFT00000007856
13	*Macaca nemestrina*	A0A2K6CXA8	ENSMNET00000052604
14	*Ursus maritimus*	A0A384D5M7	ENSUMAT00000007048
15	*Myotis lucifugus*	G1P8N8	ENSMLUT00000007177
16	*Cavia porcellus*	H0VHZ5	ENSCPOT00000011006
17	*Macaca fascicularis*	A0A2K5WLE6	ENSMFAT00000012194
18	*Papio anubis*	A0A096MZN6	ENSPANT00000011966
19	Hybrid TB	A0A4W2BW88	ENSBIXT00000010137
20	*Mustela putorius furo*	M3YUR9	ENSMPUT00000015316

Source: Prepared by the authors

5.4.2 Analysis and setting the best-fit model of DNA evolution

The sequences were selected and aligned using ClustalW (Thompson; Higgins; Gibson, 1994) using MEGAX (Thompson; Higgins; Gibson, 1994). Phylogenetic reconstructions by Bayesian Inference (BI) and Maximum Likelihood (ML) require the setting of a model DNA evolution to calculate the probabilities of nucleotide changes (Guindon et al., 2009). The “GTR+I+G” was selected as the best evolution model of *apxIA* gene by jModeltest 2 program (Darriba et al., 2012). GTR (Kirkwood, 1989), incorporates different rates for every change and different nucleotide frequencies. In addition, a proportion of invariable sites (+I) (Kirkwood, 1989) and/or rate of variation across sites (+G) (Kirkwood, 1989) can be incorporated into any model (Abadi et al., 2019; Arenas, 2015).

5.4.3 Obtention of the phylogenetic three and heatmaps

To expedite the construction of the phylogenetic tree, the alignment was trimmed using Gblocks Server Version 0.91 (Castresana, 2000) (considering parameters for a less stringent selection). Phylogenetic tree was inferred by Markov Chain Monte Carlo (MCMC) method using MrBayes v3.2.7a (Huelsenbeck; Ronquist, 2001) and phylogenetic trees were calculated in two

runs with 5,000,000 (five million) generations and a sampling frequency of 1000. The parameter convergence was analyzed and 25% of the trees generated were burned to produce the consensus tree.

In identification of the content of amino acids the Expasy ProtParam (http://www.expasy.org/tools/protparam.html) (Gasteiger et al., 2005) tool was used for different lactoferrin of mammalians and the result was plotted and analyzed and synchronized. For consolidate this information the heatmaps with amino acids content that accompany the phylogenetic trees were constructed, with later use of the software R (R, 2016) together with the gplots package in the heatmap.2 function. For this analysis was decided the focus in the difference between the reference sequence *Mus musculus* and the sequence from the animals utilized for producing milk like *Bos taurus* and *Capra hircus*.

5.4.4 Aspects of the primary sequence's analysis and secondary structure

Physicochemical properties from protein sequences were computes using Expasy ProtParam (Gasteiger et al., 2005). With this tool the acquired properties from LTF such as isoelectric point (pI), molecular weight (Mw), instability index, aliphatic index, and grand average of hydropathicities (GRAVY). For the secondary structure (which is related with protein folding), was used PSIPRED server and the CFSSP (Chou and Fasman Secondary Structure Prediction server) in analysis of helix, sheet, and turn/coil of amino acid sequences of different LTF of mammals. Ploting the results focusing on *Mus musculus*, *Bos taurus* and *Capra hircus* species (http://cho-fas.sourceforge.net/index.php).

5.4.5 Prediction of the 3D model structure

The program I-TASSER (Roy et al., 2010; Yang et al., 2015; Yang and Zhang, 2015) was used with the input of the amino acid sequences of LTF from 20 different species of mammals listed in the Table 1. This program model information about ligands, enzymes, and biological aspects of the sequences, giving one global output from each protein. When a template, normally a high sequence similarity other log or paralog, exists it can be used to create realistic structural models that allow for 3D analysis to be undertaken. For this reason, the template-based system deployed in I-TASSER was chosen for modeling of this family of proteins.

The quality of protein models obtained was further visualized and tested by Ramachandran (RM) plots. Predicted protein model of LTF from different species of

mammalians was evaluated for quality of protein models obtained of QMEAN (Benkert et al., 2011), with the Ramachandran plot using the SWISS Model server (Bertoni et al., 2017; Waterhouse et al., 2018) based on this system standards. SAVES server, with the standards ERRAT (Colovos and Yeates, 1993) and Verify 3D (Bowie et al., 1991, Luethy et al., 1992). ERRAT analyzes the statistic of non-bonded interactions between different atom types and plots the value of the error function versus position of a 9-residue sliding window, calculated by comparison with statistics from highly refined structures. The Verify 3D determines the compatibility of an atomic model (3D) with its own amino acid sequence (1D) by assigning a structural class based on its location and environment and comparing the results to good structures.

5.4.6 Model alignment and evaluation

Initially I-TASSER models were generated locally using the KAY high performance computing network, these models were not suitable for analysis as they did not contain heteroatoms of the HOLO configuration of LTF thus, Swiss-model was used with Holo- LTFs as templates for modelling. Each model was validated using PROCHECK (Laskowsk et al., 1993) (Laskowsk et al., 1993) and Molecular Operating Environment (MOE) (https://www.chemcomp.com/Products.htm) (Vilar; Cozza; Moro, 2008) to ensure homology model quality. Thereby, 20 models were created, as they are not yet released crystal structures, save for *Bos taurus* which does have X-ray crystal structures, e.g. 1BLF. The *Bos taurus* xray structures were compared with the homology model generated and they were comparable in 3D except for the carbohydrate motif which is a known PTM. To this work since the PTM patterns of other LTF species are not known it was decided that the homology model will be used. Models were loaded into the program MOE and each model prepared prior to analysis. Protein homology models were structure prepped with the following settings: Protonate3D, fix atoms farther than 8A from ligands (H close to ligands will not be fixed) and models refined to an RMS gradient of 0.1kcal/mol/A. The models were then sequence aligned using all residues, and then superimposed. RMSD (Root Mean Square Deviation) and ligand interactions analysis was then carried out.

5.4.7 Functional analysis and protein-protein interaction

I-TASSER server by COFACTOR (Zhang et al., 2017) was used to know the interaction of LTF of 20 species of mammalians with other proteins with biological aspects. The query sequence for comparison was choose based in possible industrial interest, so, the LTF from *Bos taurus, Capra hircus* and *Mus musculus*, based on that the functional protein association network was generated.

5.4.8 In silico digestion of proteins and physicochemical property prediction

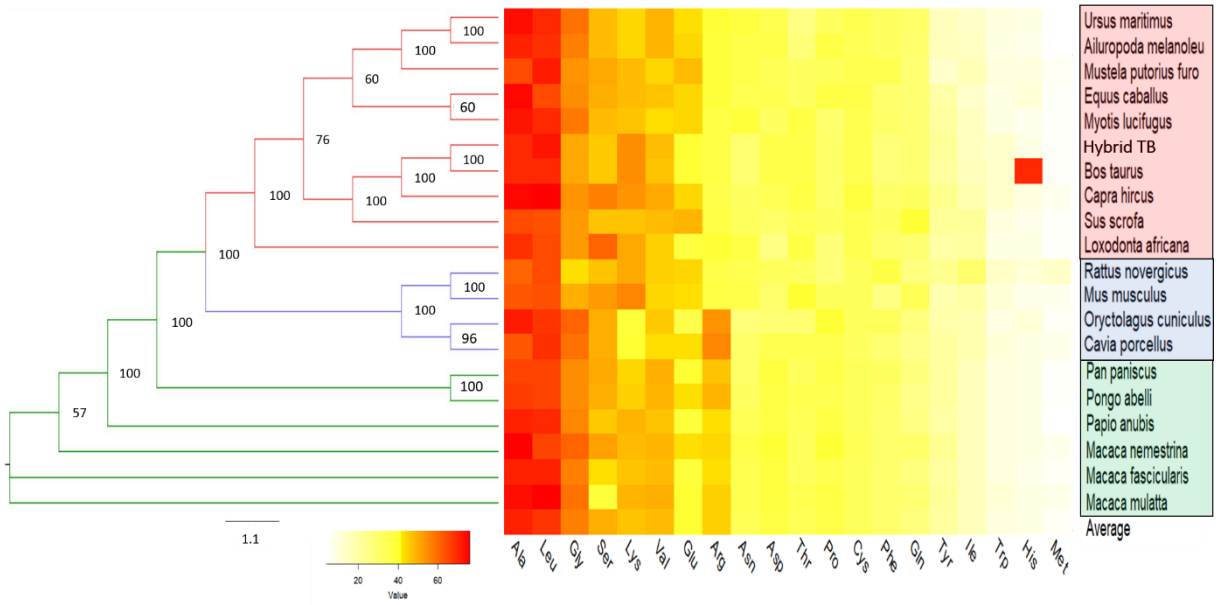
Sequences were passed through the Feptide Database (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6656964/) to stimulate peptic (pH 2.0) and tryptic digestion of the proteins producing a set of peptides based on the cleavage motifs of the enzymes, resulting digested peptides were then screened within the database for predicted bioactive peptides. The resulting peptides physiochemical properties were predicted using the ModlAMP python package (Müller *et al.*, 2017) and summary plots generated in Graphpad prism 8.

5.5 Results

5.5.1 Phylogenetic aspects of lactotransferrin and amino acids content

The phylogenetic tree and heatmap distance matrix (Figure 1) comprise the sequences of lactotransferrin from 20 species of mammalians, follow information in the Table 1. The results show three distinct monophyletic clades: red (A), blue (B) and green (C), according to identity of sequences. The clade A consists of 10 sequences belonging to ruminants' domestic animals which are important in the food production aspect and too, conservation species like *Loxodonta africana* and *Ursus maritimus*, whereas clade B (blue) consists *Rodentia* and *Lagomorpha* order sequences. Clade C (green) is composed of six sequences of primates with LTF sequences available at the databases above.

Figure 1 - Consensus tree of nucleotides sequences and heatmap distance matrix of 20 amino acids content of lactotransferrin of different species of mammals. The lines in their respective colors demonstrate: Clade A as red, Clade B as blue and Clade C as green. Hybrid TB means one animal produced with the reproduction from two species *Bos taurus* and *Bos indicus*. The clades and the content of amino acids are related, so that, the red dots demonstrate the greater amount of that amino acid and the light colors smaller.



Source: Prepared by the authors

In the heatmap, changes in amino acids from one species to another are observed in LTF protein, which may be related to mutational changes in the nucleotides. In view of that, GLU and ARG are the amino acids that separate clade A from C, with B being a miscellaneous of both. The histidine amino acid (His) for the *Bos Taurus* species stands out from the rest of the clades, as a discrepant unit in greater quantity for this protein. With the results of the Supplementary Figure 1, the Clade A the relation with one high bootstrap with *Bos Taurus* and *Capra hircus* close and how far they are from the reference sequence which we choose *Mus musculus* from the *rodentia* order.

5.5.2 Characterization of lactoferrin: chemical aspects

The Supplementary Figure 2 shows the different physiochemical properties of LTF of different species of mammals. The total number of amino acid residue of LTF was different in the species, with values variating from 704-747 (Supplementary Figure 2a). The molecular weight seems to be related with the amount of amino acids (Supplementary Figure 2a), with *Capra hircus* one considerate distance from the genus *Bos sp.* and others, and grouping with genus *Macaca* sp. In addition, the instability index (Supplementary Figure 2b) provides an estimate of the stability of the protein in a test tube, numbers above 40 indicates unstable protein

and below that is stable. In this case, with LTF sequences applied, just the mammal *Rattus novergicus* presents stable parameters following the server standards.

The LTF isoelectric point (supplementary Figure 2c) was considered neutral to strongly alkaline and the protein was considered thermostable. It has been found that almost neutral pH requires in vivo conditions in contrast to in vitro to determine optimal LTF activity (Sanchez et al., 1992). In the same Figure, GRAVY (Supplementary Figure 2d) and the aliphatic index (Supplementary Figure 2e) are related to important characteristics for milk LTF. GRAVY below the standards indicated that a better interaction was established between protein and water.

5.5.3 Secondary structure prediction

The number of amino acids directly reflect the difference aspects of helix, sheet and coil of the LTF from different species of mammalians (Supplementary Figure 3-4). In Supplementary Figure 3a is possible see the sensitive balance between the amount of helix and sheet in the species. In the Supplementary Figure 3c it`s possible analyze one carton from *Mus musculus* the reference species and *Bos taurus* and *Capra hircus*, ruminants. As follow the analysis from Supplementary Figure 3a and b, the *Capra hircus* presents differences between the other species. It is possible isolate some parts in the Supplementary Figure 3c, like the point of 100 aa (amino acids), is different in points 200 and 700 comparing all tree. But they have more similar points like the 300 and 600 between *Mus musculus and Bos taurus*, and between *Bos taurus and Capra hircus* in the point 700. The specie from the *Rodentia* order and the other from the *Artiodactyla* order show in the Supplementary Figure 3 are more proximately in the number amino acids in the content of helix, sheet and turn too. These characteristics presented are important for the protein structure and stability.

The Ramachandran plot (Supplementary Figure 4) revealed a characteristic of a good quality model, with more than 90% residues in favoured region, which characterizes a good quality model. In Supplementary Figure 5-7 it is possible visualize the percentage of those components and in almost all sequences of LTF the alpha helix it`s predominated, followed like beta-sheets. This aspect reflects on the protein stability, like for example during the heating process.

The secondary structure prediction result shown here indicated that the percentage of alpha helix (Supplementary Figure 5-7) was much greater than the percentage of other type of conformations of the protein such as sheet and turn. Percentage of beta sheet show variation in all selected organisms but seems lower in the primates like *Macaca nemestrina* and *Macaca*

mulatta. The percentage of turn show the same variation between 12 and 15%. No disordered protein binding sites were discovered (Supplementary Figure 5).

5.5.4 Structural protein modeling and analysis

For the basic comparison was used one well-known sequence from *Mus musculus* to help analyze the predicted three-dimensional structure. The sequence identity of the query sequence of the animals is presented in the Table 1. With the TM-score and the estimated RMSD (Root Mean Square Deviation) following information of the program standards (I-TASSER), the accuracy of structure modeling and with the C-score data, the model it`s reliable. A TM-score > 0.5 indicates a model of correct topology and a TM-score <0.17 means a random similarity. From the TM-score (Table 2), the numbers in this model goes from values over 0.5. This information is side by side with the C-score in Table 2. In general, a local error (e.g. a misorientation of the tail) in an average distance of all residue pairs in two structures can raise RMSD value, although the global topology is correct. In TM-score, however, the small distance is weighted stronger than the big distance which makes the score insensitive to the local modeling error.

Table 2 - I-TASSER parameters of the protein modeling in lactotransferrin of 20 species of mammals using amino acids data.

Species	Lenght	C-score*	Estimated TM-score**	Estimated RMSD(Å)
Mus musculus	707	1.15	0.87±0.07	5.6±3.5
Bos taurus	708	0.85	0.83±0.08	6.2±3.8
Capra hircus	754	0.21	0.74±0.11	7.8±4.4
Equus caballus	*715*	0.60	0.79±0.09	6.8±4.0
Sus scrofa	704	0.72	0.81±0.09	6.5±3.9
Pongo abelli	712	0.93	0.84±0.08	6.1±3.7
Pan paniscus	*711*	0.71	0.81±0.09	6.5±3.9
Macaca mulatta	747	0.66	0.80±0.09	6.7±4.0
Rattus novergicus	709	0.62	0.80±0.09	6.7±4.0
Oryctolagus cuniculus	709	0.62	0.80±0.09	6.7±4.0
Ailuropoda melanoleuca	706	0.57	0.79±0.09	6.8±4.1
Loxodonta africana	708	0.67	0.80±0.09	6.6±4.0
Macaca nemestrina	747	0.15	0.73±0.11	7.9±4.4
Ursus maritimus	708	0.82	0.82±0.08	6.3±3.8
Myotis lucifugus	710	1.09	0.86±0.07	5.7±3.6
Cavia porcellus	712	0.61	0.80±0.09	6.7±4.0
Macaca fascicularis	710	0.79	0.82±0.08	6.3±3.9
Papio anubis	710	0.94	0.84±0.08	6.0±3.7
Hybrid TB	708	0.66	0.80±0.09	6.6±4.0

Mustela putorius furo	692	1.08	0.86±0.07	5.7±3.6

Source: Prepared by the authors; **Legend:** The first column (species) it`s the identification of the mammalian was chosen based on the sequence of lactotransferin. The second until the fourth column are results from the modeling from I-TASSER related with standards produced the program using his own mathematical and statistical analysis. * C-score>-1.5 indicates a model with correct topology, information by I-TASSER website; **Is a metric for measuring the similarity of two protein structures, scores below 0.17 correspond to randomly chosen unrelated proteins, information by I-TASSER website.

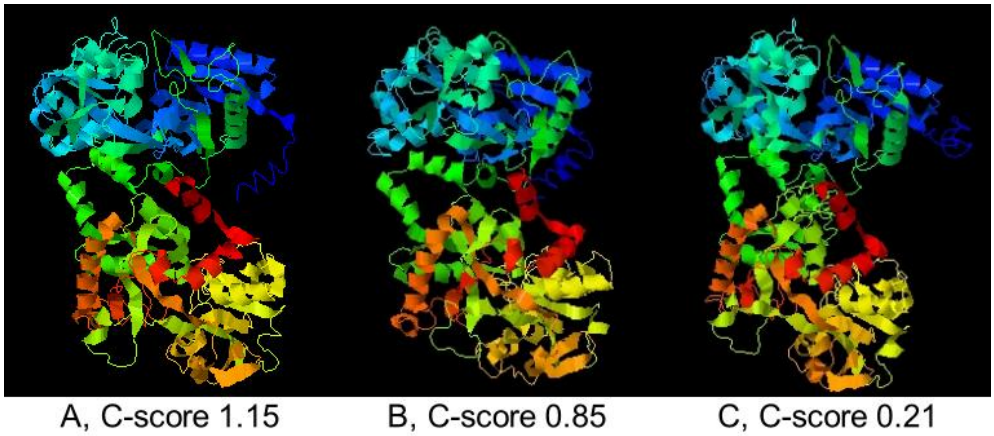
The predicted protein modelof the query sequence was analyzed for model evaluation and validation. The QMEAN scores for each homology model were satisfactory for structural analysis. In the residues showing a score below 0.6 are expected to be of low quality, and as the standards of the modeling program (SWISS model), different chains are shown in different colors. In this Figure, the results from *Mus musculus* (G), *Bos taurus* (H) and *Capra hircus* (I) shows the quality of the model, average value, so the homology of the model with this analysis it`s equal with the values from Verify 3D and ERRAT giving average and reliable models.

Orthogonal quality checks of homology models through SAVES and ERRAT confirmed models were appropriate for analysis with respective results of >90% and 83-94%. For crystal structures refined high resolution structures generally place >95% with lower resolution (2.5 to 3Å) images scoring between 90-90%

The 3D model from the *Mus musculus*, *Bos taurus* and *Capra hircus* in comparison presents similarities, however, the *Capra hircus* results from the amino acid alignment from the data show a larger sequence of LTF nucleotides and amino acids (Supplementary Figure 5). In addition, *Capra hircus* amino acids sequence is close for the model from the *Bos taurus* family (Supplementary Figure 5), as shown in the Consensus tree (Figure 1). There is 46 amino acids difference in the goat protein in relation to other species. Other information from that animal model it`s one interspace between the lobes, that is interesting from a structural point of view.

Following that, the C-score (Table 2) is a confidence score for estimating the quality of predicted models by I-TASSER. This score is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C-score is typically in the range of [-5.2] and the C-score of higher value means a model with a high confidence and vice versa. The values from this model (0.15 to 1.15) described in the Table 2 are considered as average values since the range it`s considerable variated. It is possible see that in the (Supplementary Figure 9) from the *Mus musculus* (A), *Bos taurus* (B) and *Capra hircus* (C). The average C-score for binding sites are presented in Supplementary Figure 9. As the results of the binding sites, all the query sequences present serine (Ser) and aspartate (Asp) portions (Supplementary Figure 13) and no enzymatic portions were presented in modeling using I-TASSER.

Figure 2 - Visualization of the 3D structure from A (*Mus musculus*), B (*Bos taurus*) and C (*Capra hircus*) of lactotransferrin from I-TASSER, with the respectives C-scores below each figure.

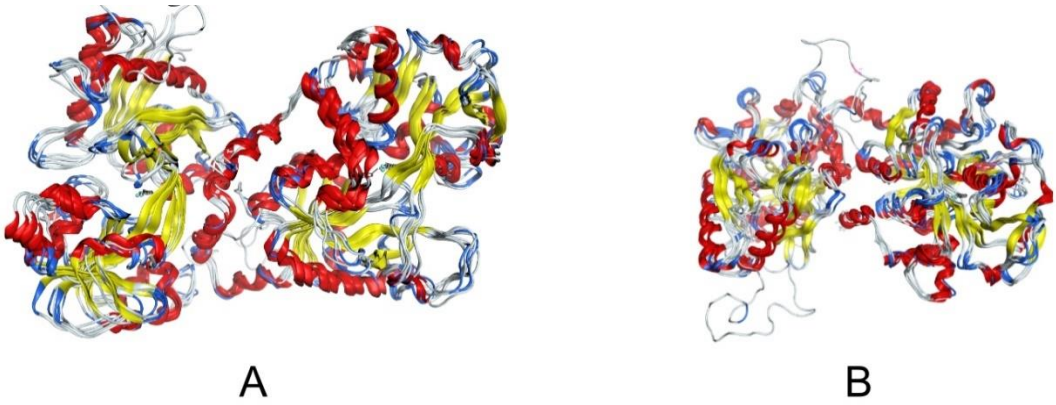


Source: Prepared by the authors

5.5.5 Model interactions and evaluation

The overall structural architecture of the 20 proteins is highly conserved, in the Figure 3. The two major deviations in the overall 3D structure are found in the *Capra hircus* and *Macaca mulatta* model and these two structurally deviate from the other species from what appears to be insertions of 46 (position 406-450) and 37 (position 295-331) amino acids (AA), respectively.

Figure 3 - A: Structural alignment of 20 models of Lactoferrin showing high structural alignment amongst the models B: Structural alignment of 20 models of Lactoferrin what presents two deviations from model 3 and 8, top loop and bottom loop, respectively.



Source: Prepared by the authors

The *Capra hircus* model showed an insertion of 46 AA into the interlobar space between the C and N terminals of the protein, while the *Macaca mulatta* model showed an insertion of AA into the exterior lobe. Although both variances in the structural trend are interesting, the *Capra*

hircus was chosen to carry out more analysis due to the scalability of goat milk compared with samples of other mammals.

It has been shown that both human and bovine milk LTF exhibit serine protease activity, which has been assigned to the Lys73 and Ser259 of human lactoferrin (Hendrixson, et al., 2003) (Supplementary Figure 13). These amino acids are present in these species and are structurally conserved which indicates that the proteolytic activity should be maintained throughout the LTF species here within. Focusing on the resemblance of the models (Figure 4) presented for the species *Capra hircus* and *Macaca mulatta*, structurally deviation from the other in the position 406-450 and position 295-331 of amino acids respectively. To the iron-binding sites within LTF, all models have the same iron-binding motif (data not showed) which is characteristic of the wider TF superfamily present. The results of the resemblance of *Bos taurus* and *Capra hircus* are present in the comparison of the 3D structure modeling (Figure 4).

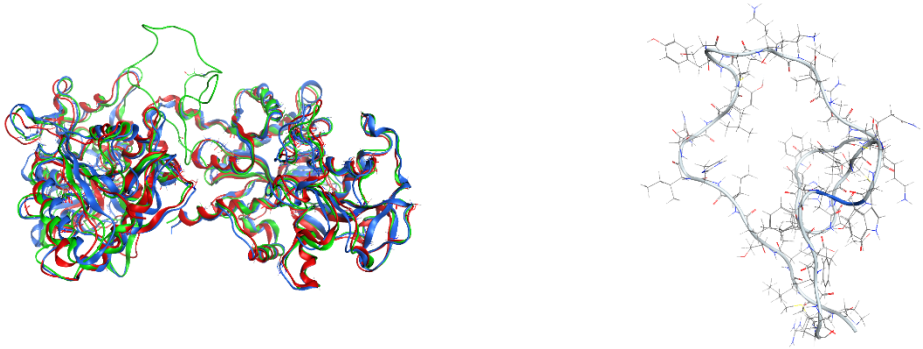
5.5.6 Functional analysis

All the sequences show the same biological prediction when the analysis its settle for the COFACTOR program in the I-TASSER server. As expected, the biological aspects related for the modeling are correlated with literature data (Niaz et al., 2019; Liu et al., 2020), since LTF has iron transport regions and antibacterial activity.

5.5.7 Peptide analysis and *in silico* digestion

Comparison of the three species (*Mus musculus*, *Bos taurus* and *Capra hircus*) (Supplementary Figure 11-13) shows a single major structural difference, in the interlobar space of the protein. It is caused by an extension of the *Capra hircus* amino acid primary structure shown below. BLAST and VAST+ searches of this region showed no significant similarities to any know protein other than *Capra hircus*, in the NCBI, MMDB (Molecular Modelling Database) or PDB (Protein Databank) Databases. More refined modelling of this region failed to show any homologous matches that would lead to a greater understanding of the biological rational for this extension.

Figure 4 - A: Structural alignment of *Mus musculus* (Red), *Bos taurus* (Blue) and *Capra hircus* (Green). B: The extended loop region of the *Capra hircus.* The green area explains the difference in the related species.



A B

Source: Prepared by the authors

5.6 Discussion

LTF along the evolutionary chain of mammals, may be undergoing adaptive selections without changing their biological function, to continue playing an important role in the development of species in the face possible of the need to adapt to diets and the environment of these animals. LTF may have “coevolved” along with mammalian defense systems, acts as a critical component in mediation of immune response, especially for coordinated interactions between innate and adaptive components and associated responses (Actor; Hwang; Kruzel, 2009).

Everything that does not change or becomes something trace or disappears. Thus, the protein remains present in the animals' genome because it is essential to them. It is possible to see in the tree that there are differences in the evolutionary scale. In addition, the bootstrap values of the phylogenetic tree are good for this type of analysis, since bootstrap values of 95% or greater be considered statistically significant and indicate “support” for a clade; alternative nodes can be rejected if they occur in less than 5% of the bootstrap estimates (Felsenstein, 1985), which means that the node is well supported in this study. This same phylogenetic tree can be viewed independently in the supplementary Figure 1.

Using the I-TASSER was possible do some observation about the models, they seem pretty similar, so having those results from the goat model, open access for new studies and more focus in the LTF, from this animal. In one research about modeling the heat shock protein 70 (HSP70) and heat shock factor-1(HSF1) proteins in water buffalo, and they find in the comparative analysis of the HSP70 protein revealed certain significant variations, but most of the sequences were found to be conserved across species (Singh et al., 2019). In the

phylogenetic tree, have three major clades, Clade A as red, Clade B as blue and Clade C as green, and those clades lactoferrin is preserved among groups of mammals, like *Bos taurus, Capra hircus*. With the sequence models of the phylogenetic tree in the clade C, all the animals are close, because all of them are primate's non-human, and the perspective of the protein conservation between species is expected.

Another result from the phylogenetic aspect it`s about the clade B (blue), since it is confirmed by the three the proximity between those species, and they present close results forward in the analysis. In this clade, *Rattus novergicus* and *Oryctolagus cuniculus* close with great bootstrap values. Since was used just one sequence of each, more rodent and lagomorph sequences are needed to clarify the picture. Some other research evaluating the lactoferrin between species has the same results and they focus and more analysis to understand more this kind of results (Lambert at al., 2005). Lambert et al. (2005) have some discrepancies in the analysis of lactotransferrin from camel and others from the same family, but in those results, the *Equus caballus* and *Myotis lucifugus* still present reliable bootstrap values in the three.

With the phylogenetic tree, opens the opportunity the understand the evolution of the amino acid content. In Figure 1 is possible to see the percentage of the amino acids and the correlation in the Clade A as red, Clade B as blue and Clade C as green. The species share high values of the amount of Alanine and Leucine and one average amount of glycine. Amounts of serine, lysine, and valine and higher in the proteins from Clade A and Clade B. Each amino acid has an important participation in the protein construction, maybe reflecting in the quality of the product. Maybe these amino acids are altering the constitution of the lactoferrin protein may be directly inferring in the conformation of it and exposing some active site or not.

The number of amino acids can directly affect the protein physicochemical aspects of molecular weight, instability index, isoelectric point, GRAVY, and aliphatic index. Was realized the comparison of those parameters with the total number of amino acids, for the molecular weight species like *Capra hircus* and *Macaca mulatta* present some differences for being farther from the others. The lactotransferrin protein sequence of the *Rattus norvegicus* using Expasy ProtParam prediction seems the one sequence with the stable label. All the other species sequences have valued over 40, which makes them in the instability index label unstable.

These parameters can suffer influence of environmental factors, in situations like autoproteolysis of proteins, and about the broader application of the instability index (II) method for the prediction of protein stability under *in vitro* conditions. Gamage et al. (2019) concluded, that method can be questionable as the stability of the protein may be dependent not only on the intrinsic nature of the protein but also on the conditions of the protein. Besides that, this type

of analysis has the confluence in how meaningful can be understand about chemical aspects of protein, for example, if the protein has the same pI from casein, the industries need use more resources to extract LF, making the process more expensive, and with those information's, the focus is avoid that.

Another aspect is the GRAVY, it`s a sum of all hydropathicity values of all amino acids divided by the number of residues in a sequence. Lactotransferrin sequences of *Capra hircus* and *Rattus norvegicus* are closer to zero. A lower range of GRAVY indicated that better interaction can be established between protein and water (Verma et al., 2016; Pramanik et al., 2017).

Another important factor for protein structure is the modeling aspects of the secondary structure aspects, as an alpha helix, beta-sheets, and turn or coil. The differences in the secondary structure are very determinant, and even the proteins are from the same family, and some of the species are close, understand this kind aspects influence the overall analysis of final products, like powder milk. For example, the secondary structures of protein in the whole milk powder began to change at 70°C and in the infant formula at 50°C, the same paper show about the β-sheet and β-turn structures in the whole milk powder both decreased in the range of 70 to 85°C, so the results imply that heating may induce the transformation from β-sheet to β-turn and with that the characterization of the product (Ye et al., 2017). Observing the data and the results, the prediction of secondary structural elements was vital for detecting conformational changes within the protein of interest (Roy et al., 2015).

The 3D structures in this research present some similarities, the research about modeling of proteins are having more space, and for proteins like lactotransferrin, the references are a bit outdated, but some basic standards seem to be present, showing less evolution of the animals since the first publications from 1990. In the lactotransferrin research, as the literature discuss, all of "transferrin family" proteins have the same basic polypeptide fold, comprising two homologous globular lobes (N-lobe and C-lobe), each further divided into two (N1 and N2, C1 and C2) domains (Aisen and Harris 1989, Baker 1994). Some authors already show information's about the amino acid sequences for the LF of eight species (human, bovine, buffalo, camel, goat, horse, mouse, pig), they discovered ~ 70% sequence identity (human/mouse 70%, human/bovine 69%, mouse/bovine 63%), consistent with the strong conservation of their 3D structures (Baker and Baker, 2004).

The idea of understanding more about the structure of LF, those results have shown that for each model, generalized correlations among residues, each partitioned into sidechain and backbone parts, were qualified as reliable in terms of mutual. This data first provides a general overview of the LF simulation results. The I-TASSER models presented enough C-score (-5,2

standards) to be considered reliable models, and when compares the species like *Bos taurus* and *Capra hircus*, similarities are presented.

For this study, the three species of the highest interest are though with the most potential for industrial use and commercial research. Since that, the biological aspects don't present changing whit the modeling analyzed here by various aspects and programs, such as from I-TASSER and SWISS model. An important consideration when considering in new uses and applications of biologically sourced material is the industrial scalability. As an example, *Macaca mulatta* although a potentially interesting LTF would not be suitable for scale up and purification via traditional methods, i.e. purification from milk, unless by genetic engineering it is possible to select constituents of milk to be produced in another mammal, e.g. the milk cow. Moreover, another interesting member of the lactoferrin family from *Capra hircus* milk could be scaled, in fact both goat herds and milk production has been on the rise since the 1960's (Miller, Lu, 2019).

All the biological aspects are presented with the same. The literature discusses those aspects and this modeling process brings light to the aspects close to the literature (Vogel, 2012; Ma et al., 2017). For example, LF can transport iron in the gastrointestinal tract, and this protein regulates the quantity of iron absorbed in the intestine (Hao et al., 2019). The iron-binding regions are found at the N- and C-terminals of the molecule, which has a high affinity for iron and can bind it reversibly to maintain the absorption and use of iron in the duodenum at a large pH range (Hao et al., 2019). It`s sugested too, LTF can present besides the antimicrobial aspect, nutraceutical, immunomodulation protective effects and anticancer activity (Giansanti et al., 2016). Those aspects bring more solid information's about LF and make with the model and the chemical and biological analysis present results which can be used in applied research.

5.7 Conclusion

This study demonstrated that lactoferrins showed high genetic similarity between some species genera and still preserved, such as domestic ruminant mammals (cows and goats) and other domestic mammals such as non-human primates. The amounts of amino acids present proportionally similar, with the exception *Capra hircus* model showed an insertion of 46 amino acids, that don`t affect the biological and chemical profile of the protein. In the *in silico* digestion the iron insertion is preserved in the species. The chemical results present models of protein with research and manufacturing potential. Understanding more about lactoferrin contributes with more data for dairy culture and veterinary medicine.

5.8 Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and the writing of the manuscript.

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APÊNDICE A - “Referências da Introdução Geral”

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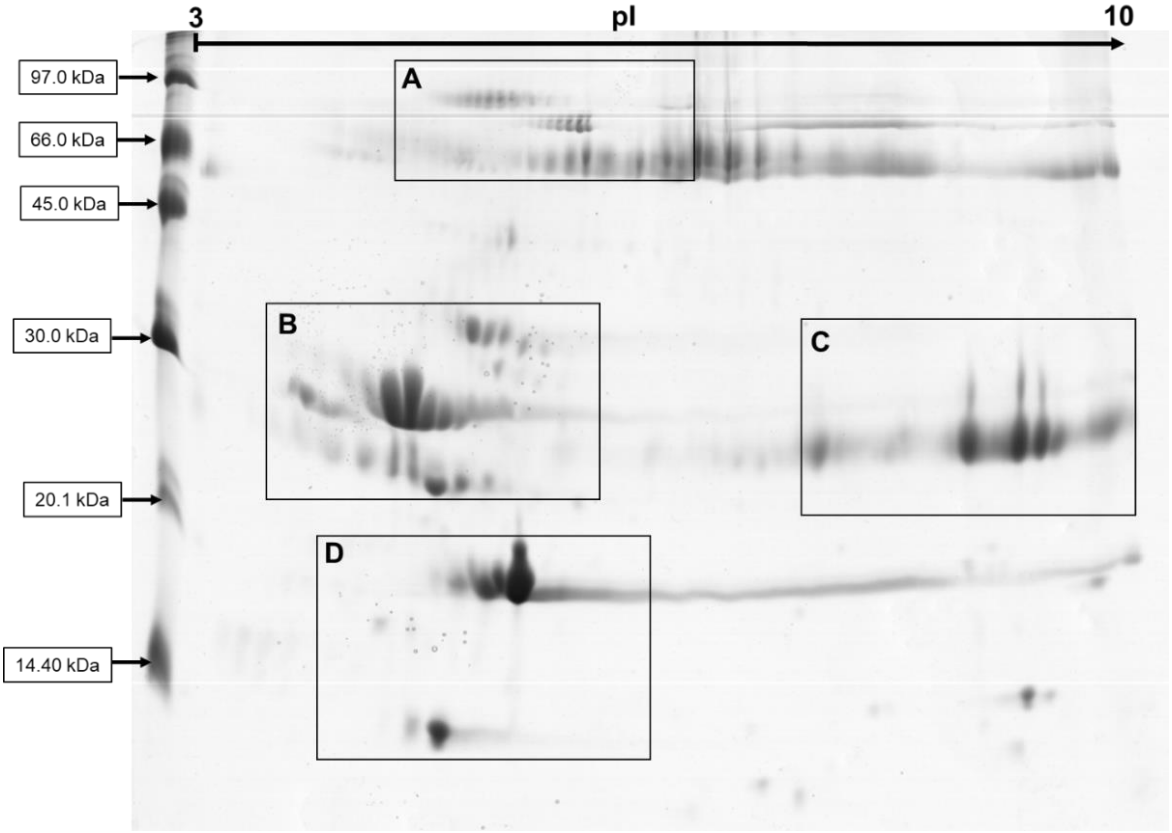
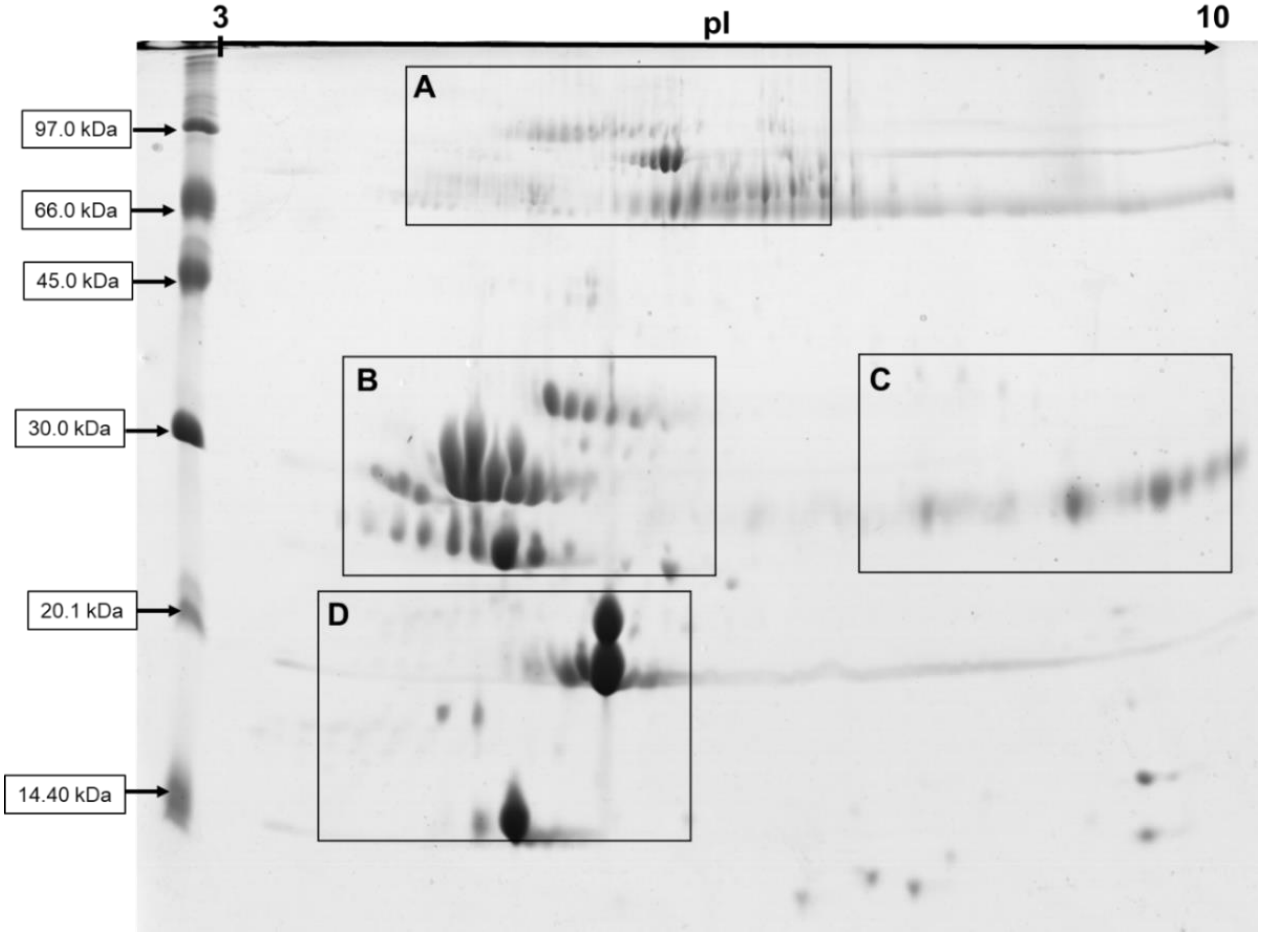
APÊNDICE B - Arquivo Suplementar ao Capítulo 1

Supplementary Figure 1 - 2D-PAGE gel scanned of protein groups (G1-G2) in the colostrum/milk from goats Representation of the moment immediately after birth (M0), A, B C and D are possible groups of proteins with different MW and pI.



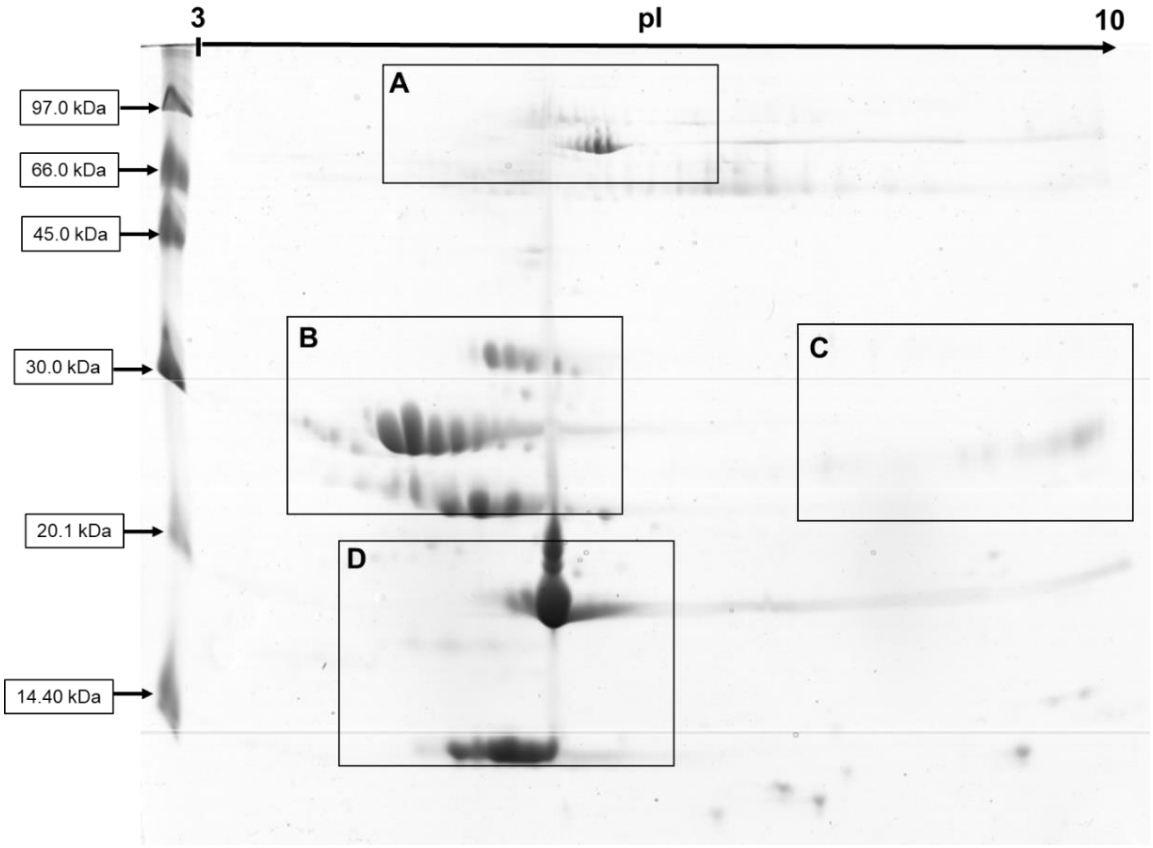
Source: Prepared by the authors

Supplementary Figure 2 - 2D-PAGE gel scanned of protein groups (G1-G2) in the colostrum/milk from goats Representation of the moment with 3 days (M3), A, B C and D are possible groups of proteins with different MW and pI.



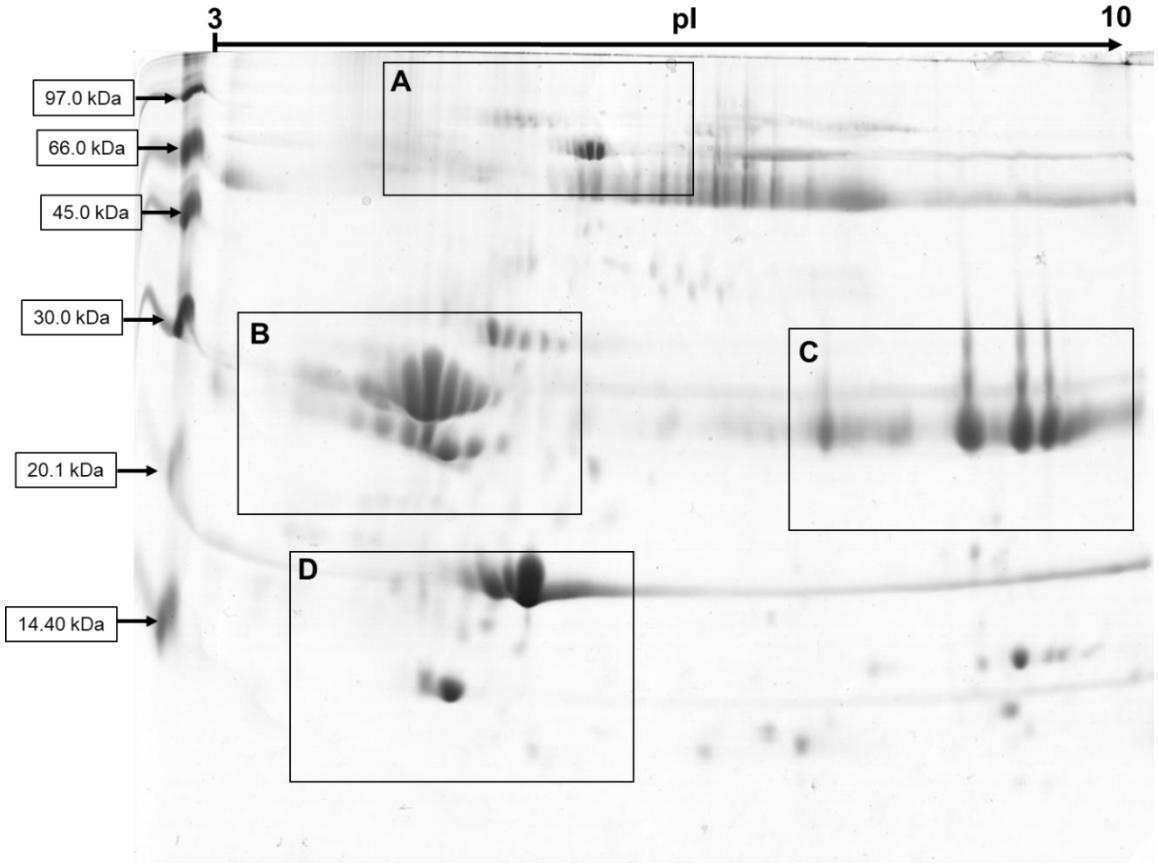
Source: Prepared by the authors

Supplementary Figure 3 - 2D-PAGE gel scanned of protein groups (G1-G2) in the colostrum/milk from goats Representation of the moment with 9 days (M9), A, B C and D are possible groups of proteins with different MW and pI.



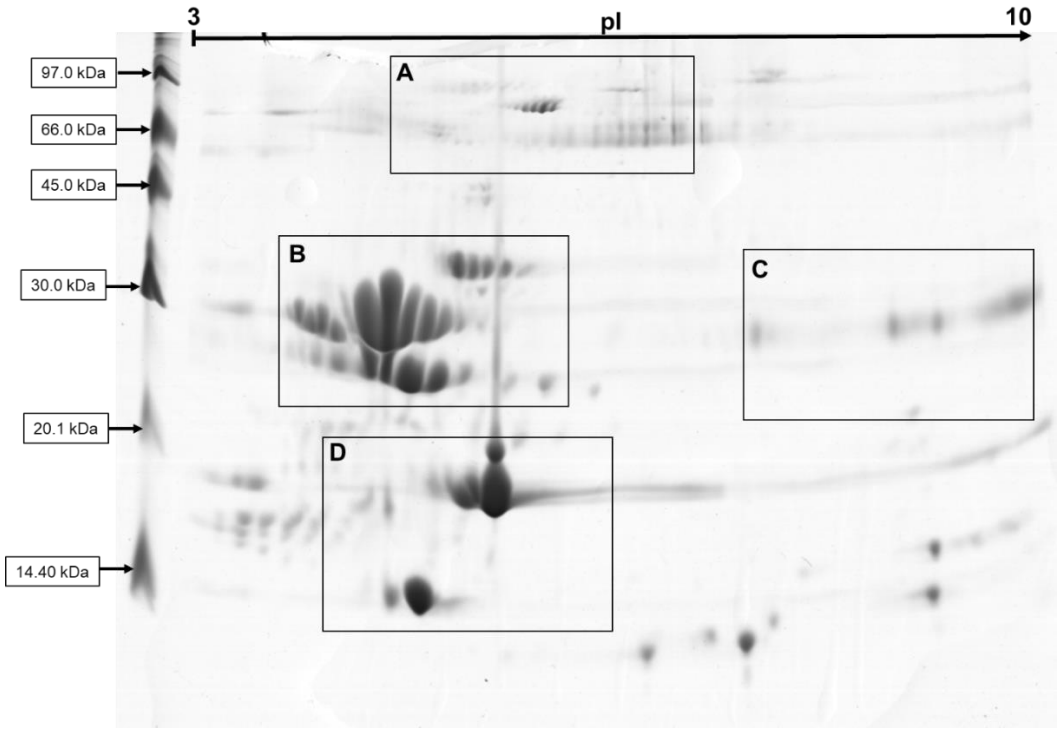
Source: Prepared by the authors

Supplementary Figure 4 - 2D-PAGE gel scanned of protein groups (G4-G5) in the colostrum/milk from goats Representation of the moment immediately after birth (M0), A, B C and D are possible groups of proteins with different MW and pI.



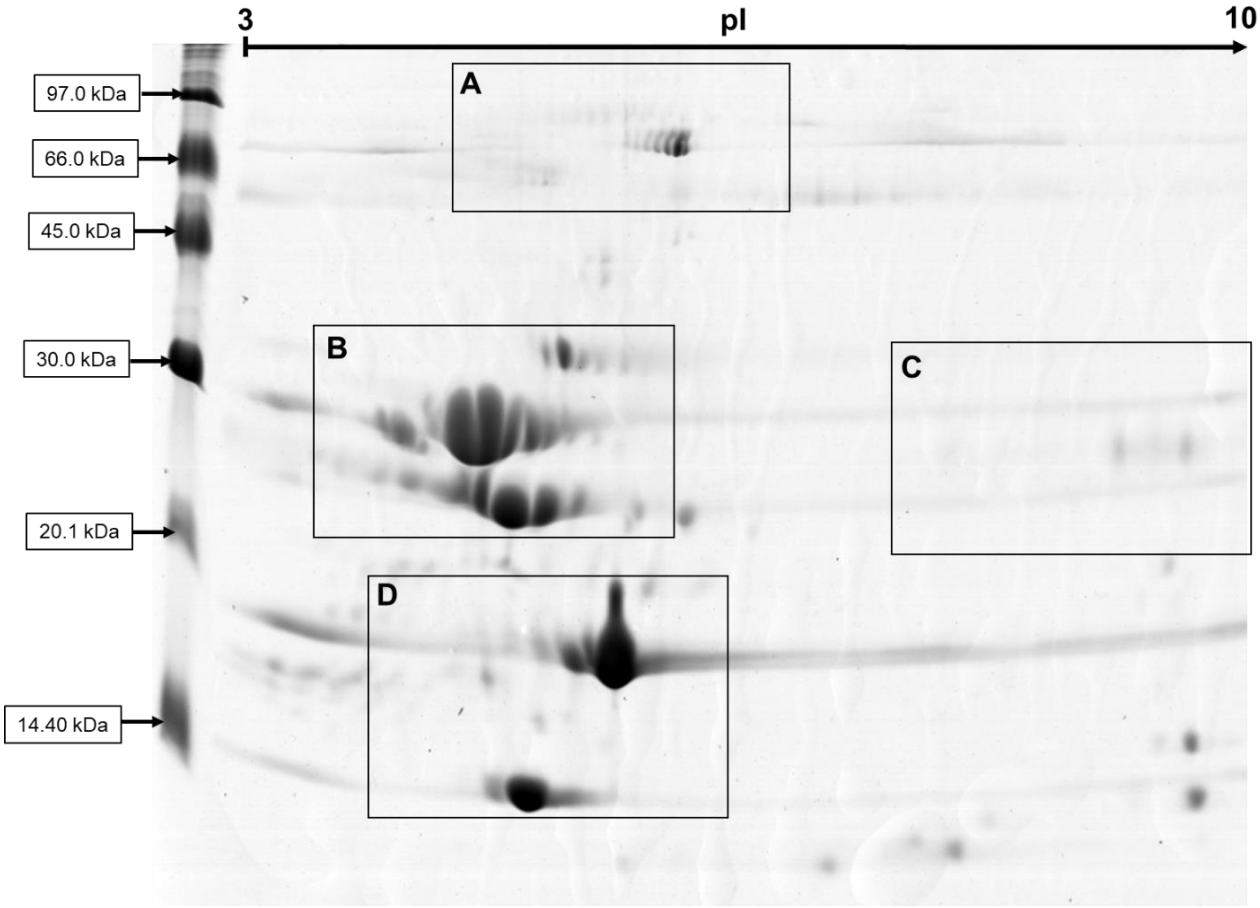
Source: Prepared by the authors

Supplementary Figure 5 - 2D-PAGE gel scanned of protein groups (G4-G5) in the colostrum/milk from goats Representation of the moment with 3 days (M3), A, B C and D are possible groups of proteins with different MW and pI.



Source: Prepared by the authors

Supplementary Figure 6 - 2D-PAGE gel scanned of protein groups (G4-G5) in the colostrum/milk from goats Representation of the moment with 9 days (M9), A, B C and D are possible groups of proteins with different MW and pI.



Source: Prepared by the authors

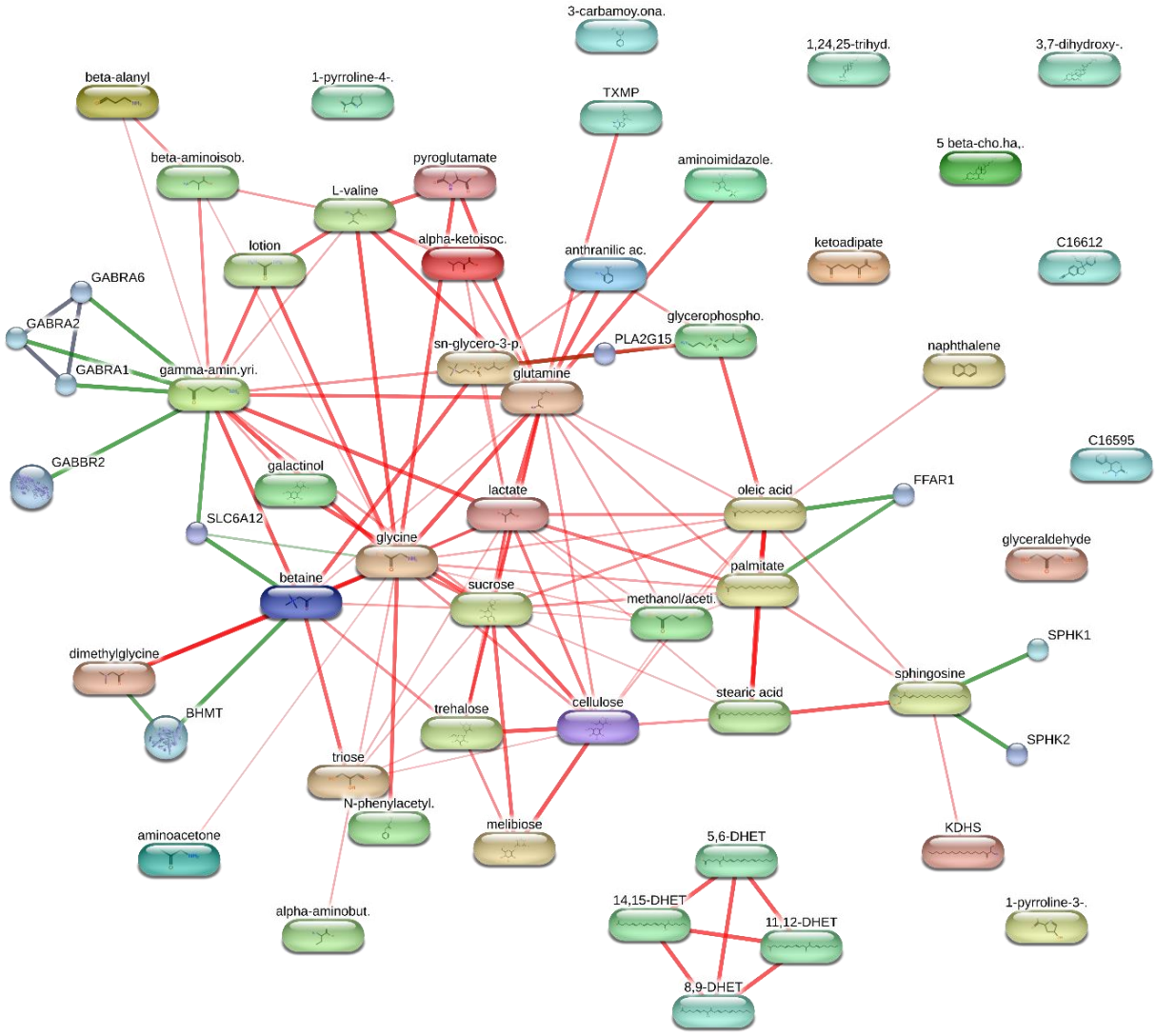
APÊNDICE C - Arquivo Suplementar ao Capítulo 2

Supplementary Table 1 - Metabolic pathways using mummichog present in the whey of goat's milk us (p<0.05).

Metabolic pathways
Citrate cycle (TCA cycle)
Arginine biosynthesis
Purine metabolism
Alanine, aspartate and glutamate metabolism
D-Glutamine and D-glutamate metabolism
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis
Glycerophospholipid metabolism
Ether lipid metabolism
Butanoate metabolism
Biosynthesis of unsaturated fatty acids

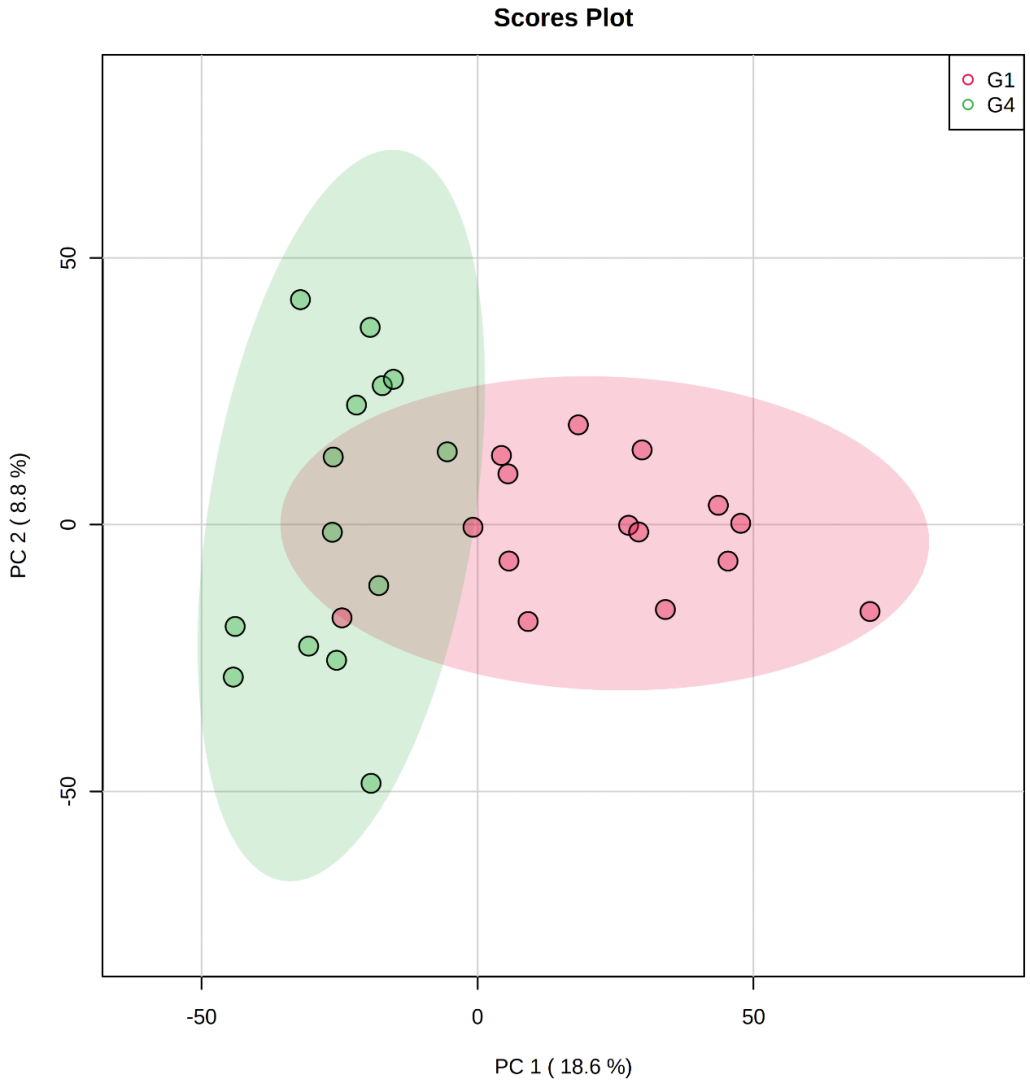
Source: Prepared by the authors

Supplementary Figure 1 - Mass-spectrometry analysis with differential metabolites and the interactions obtained from MetaboAnalyst (p<0.05). The metabolites are represented as nodes and they were classified using KEGG number in Milk Metabolome Data Bases.



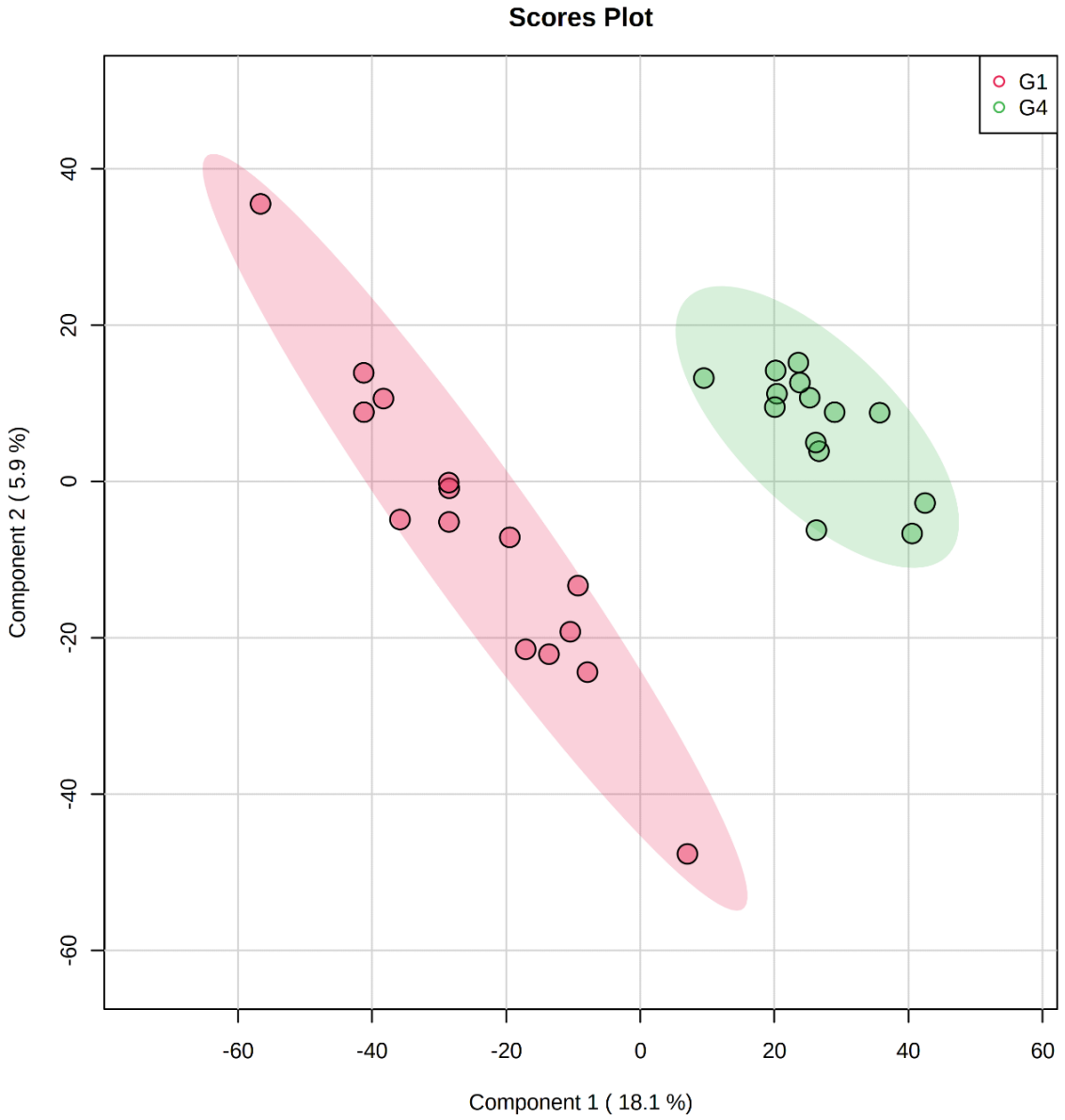
Source: Prepared by the authors

Supplementary Figure 2 - PCA scores plot between the selected first birth group (G1) and the group with more than three births (G4), the explained variances are shown in brackets.



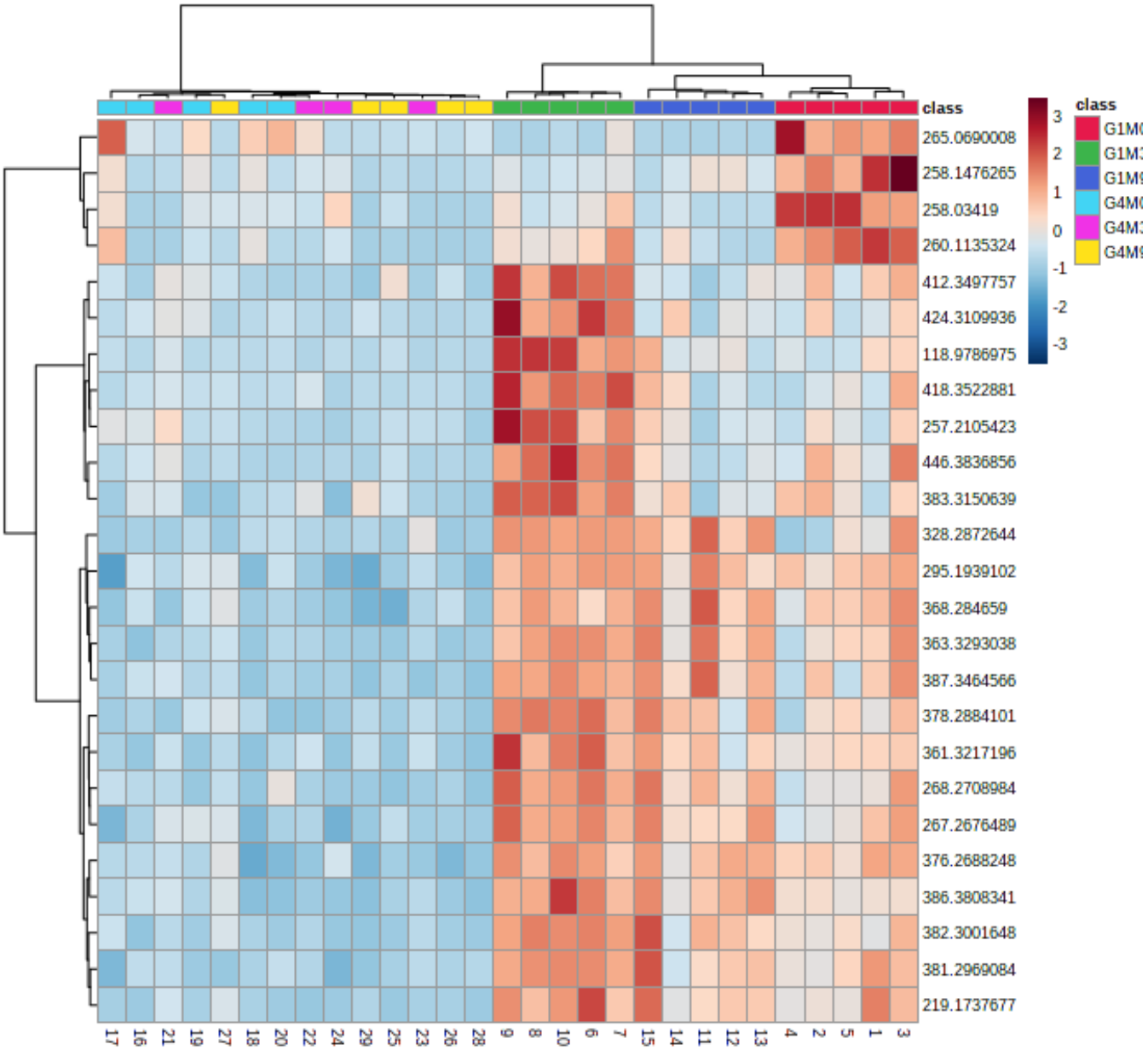
Source: Prepared by the authors

Supplementary Figure 3 - Scores plot of the PLS-DA analysis between selected components from the first birth group (G1) and the group with more than three births (G4), the explained variances are shown in brackets.



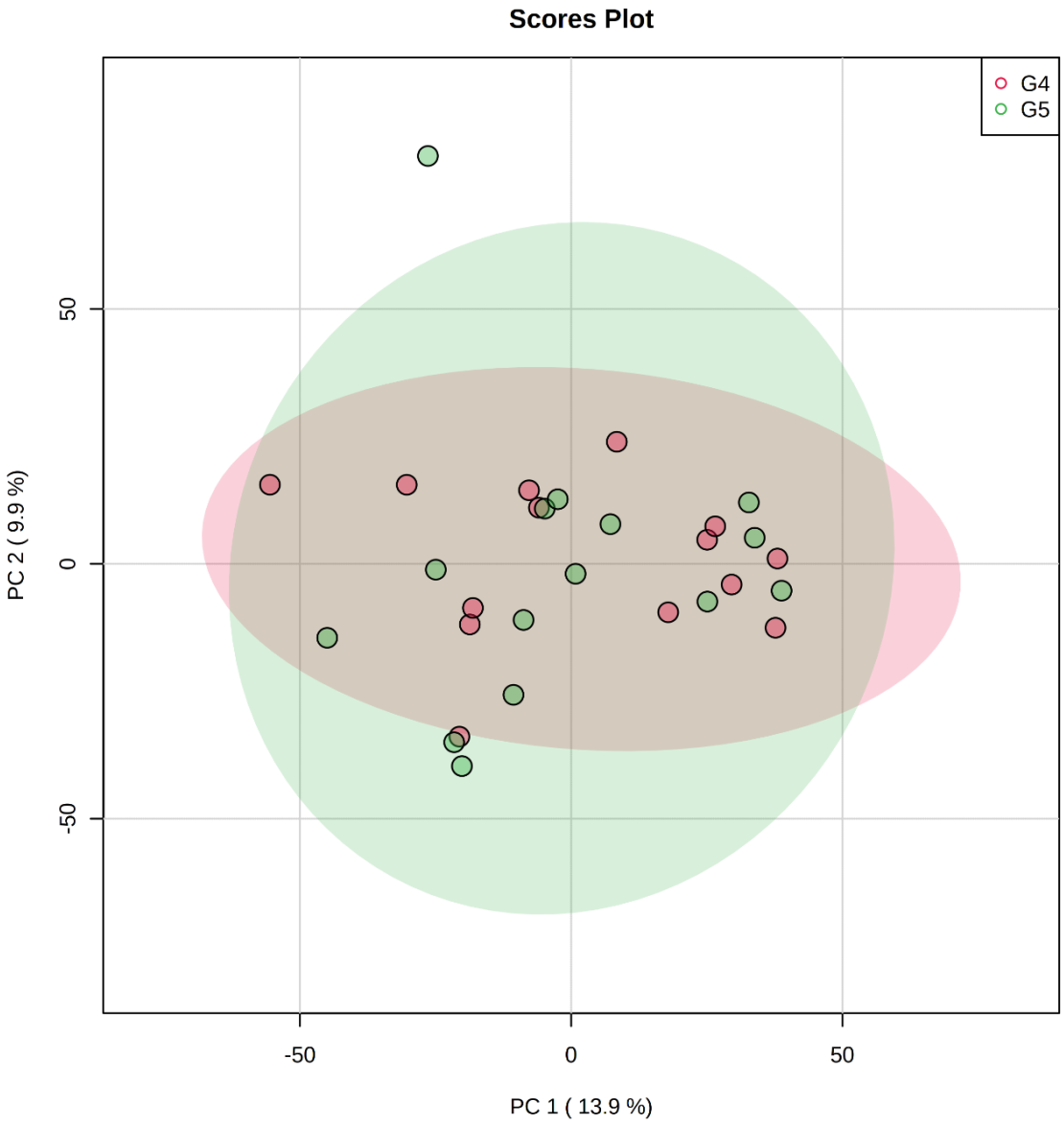
Source: Prepared by the authors

Supplementary Figure 4 - Variations and heatmap of differential features of possible metabolites using multivariate ANOVA from the first birth group (G1) and the group with more than three births (G4). Red represents positive correlation, blue represents negative correlation, and the blank portion represents p > 0.05, the colored portion represents p< 0.05).



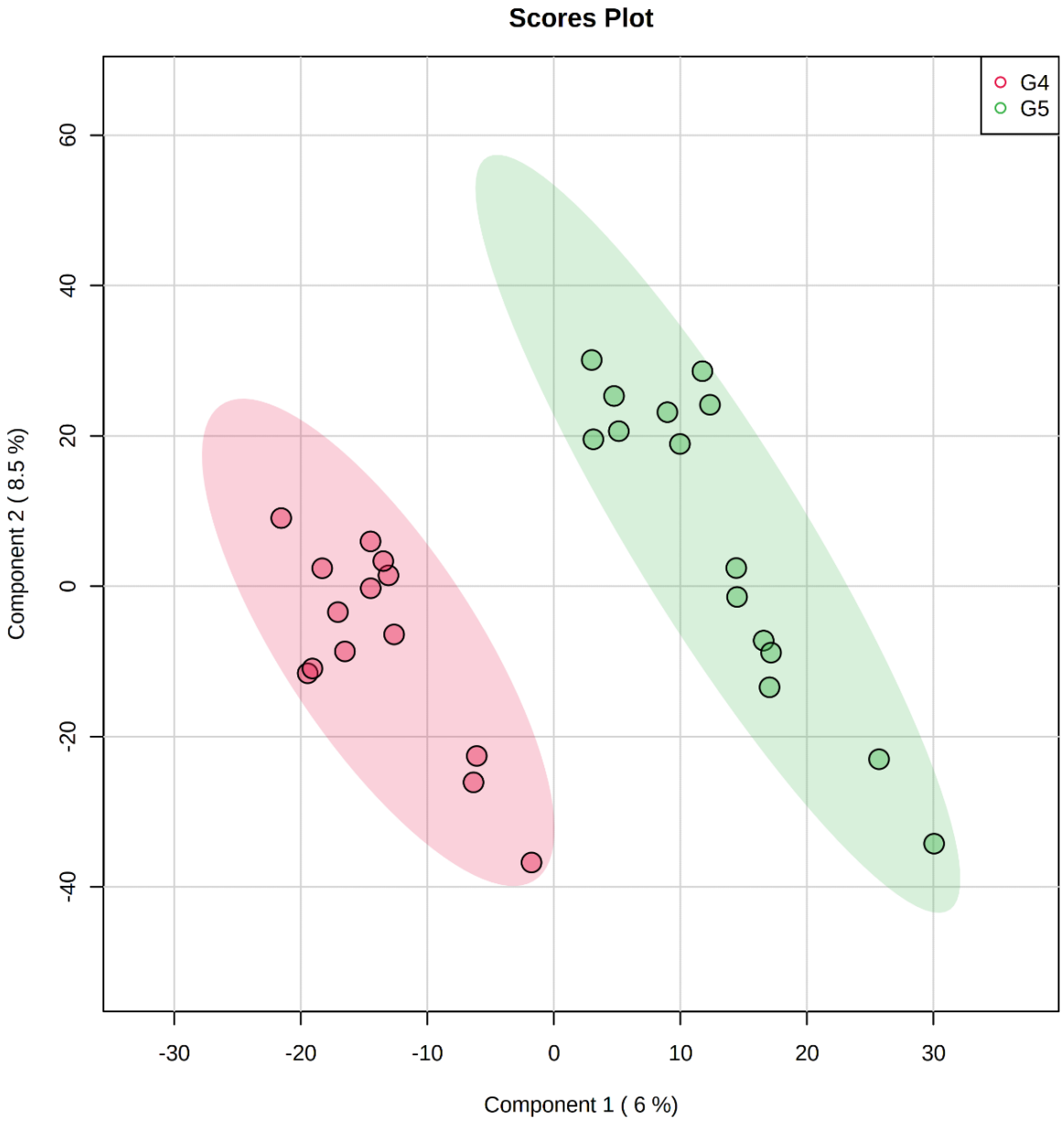
Source: Prepared by the authors

Supplementary Figure 5 - PCA Scores plot between the selected group of CAEV negative animals with more than three births (G4) and the CAEV positive group with more than three births (G5), the explained variances are shown in bracket.



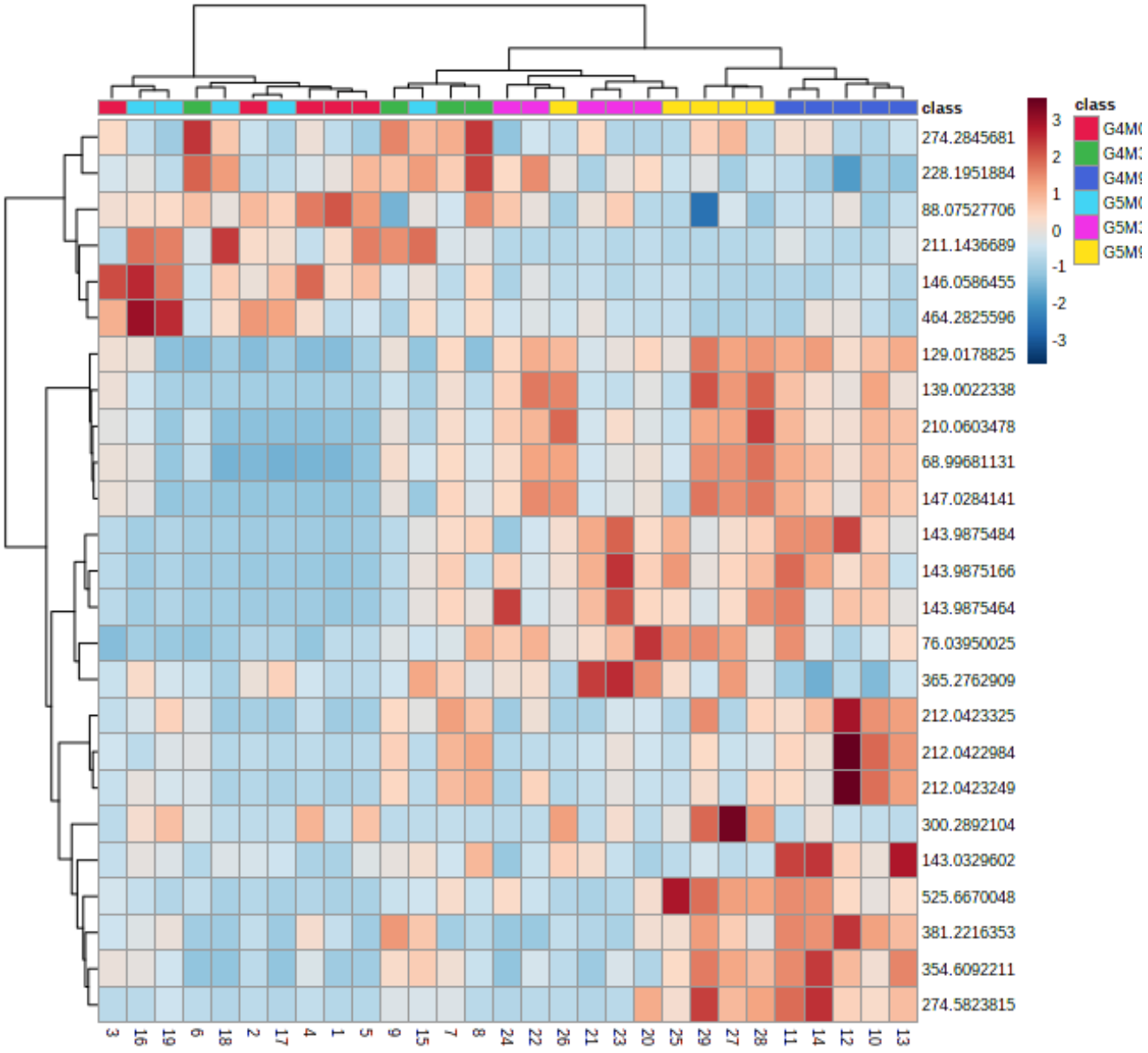
Source: Prepared by the authors

Supplementary Figure 6 - Scores plot of the PLS-DA analysis between selected components from the selected group of CAEV negative animals with more than three births (G4) and the CAEV positive group with more than three births (G5), the explained variances are shown in brackets.



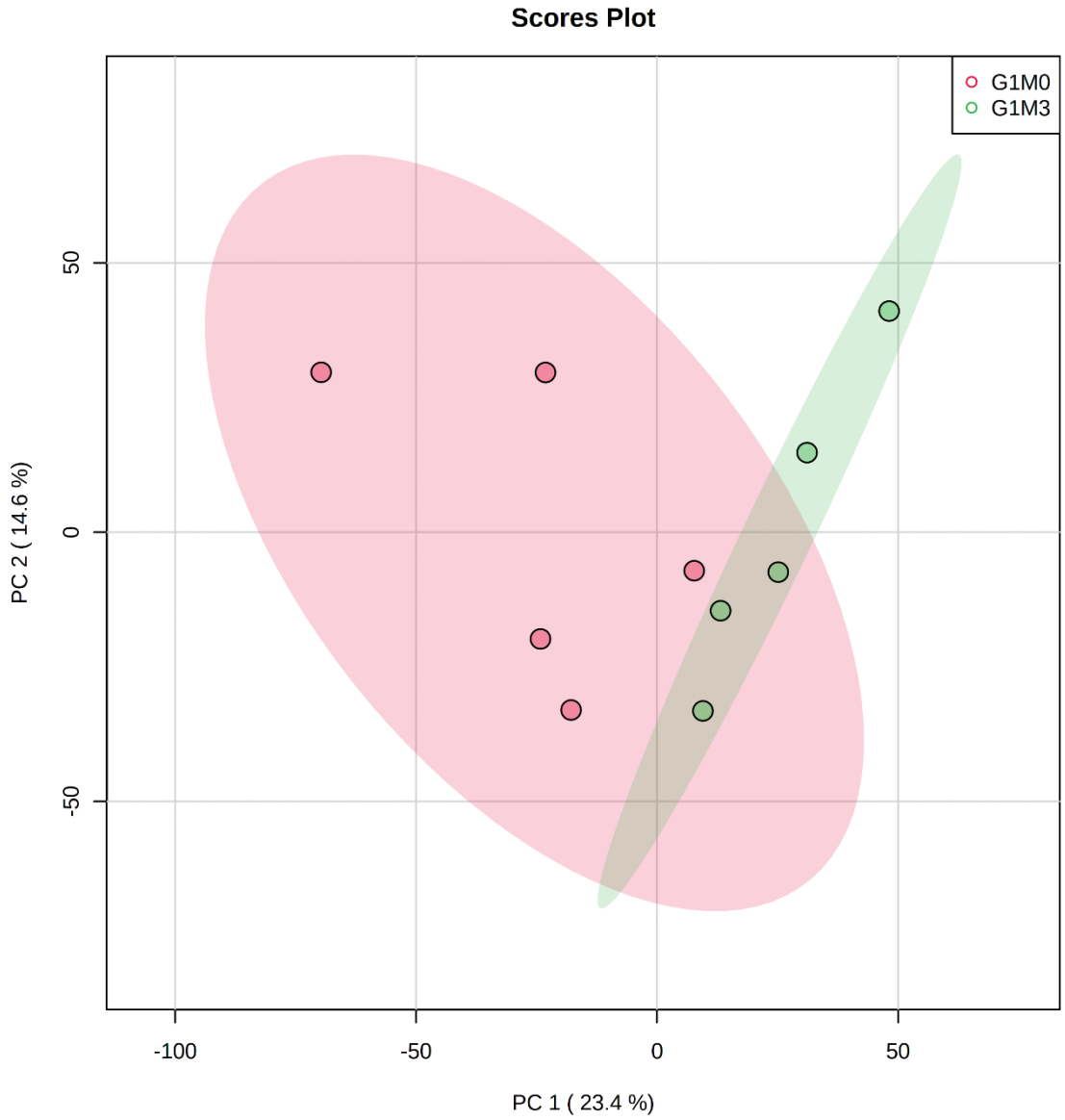
Source: Prepared by the authors

Supplementary Figure 7 - Variations and heatmap of differential features of possible metabolites using multivariate ANOVA from group of CAEV negative animals with more than three births (G4) and the CAEV positive group with more than three births (G5). Red represents positive correlation, blue represents negative correlation, and the blank portion represents p > 0.05, the colored portion represents p< 0.05).



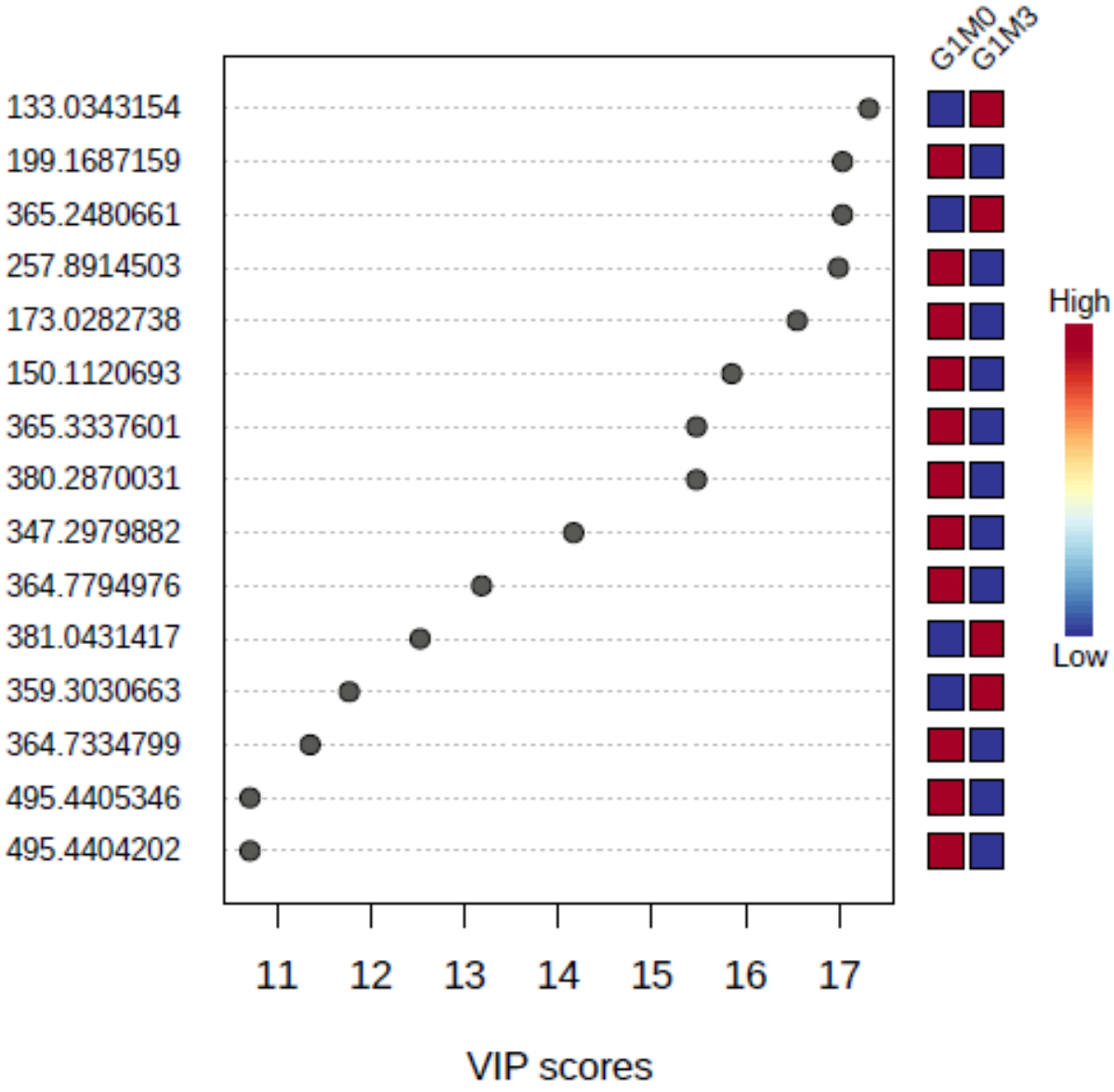
Source: Prepared by the authors

Supplementary Figure 8 - PCA scores plot with the comparison between the data from the first birth group (G1) in different postnatal moments, immediately after birth (M0) and with 3 days (M3), the explained variances are shown in brackets.



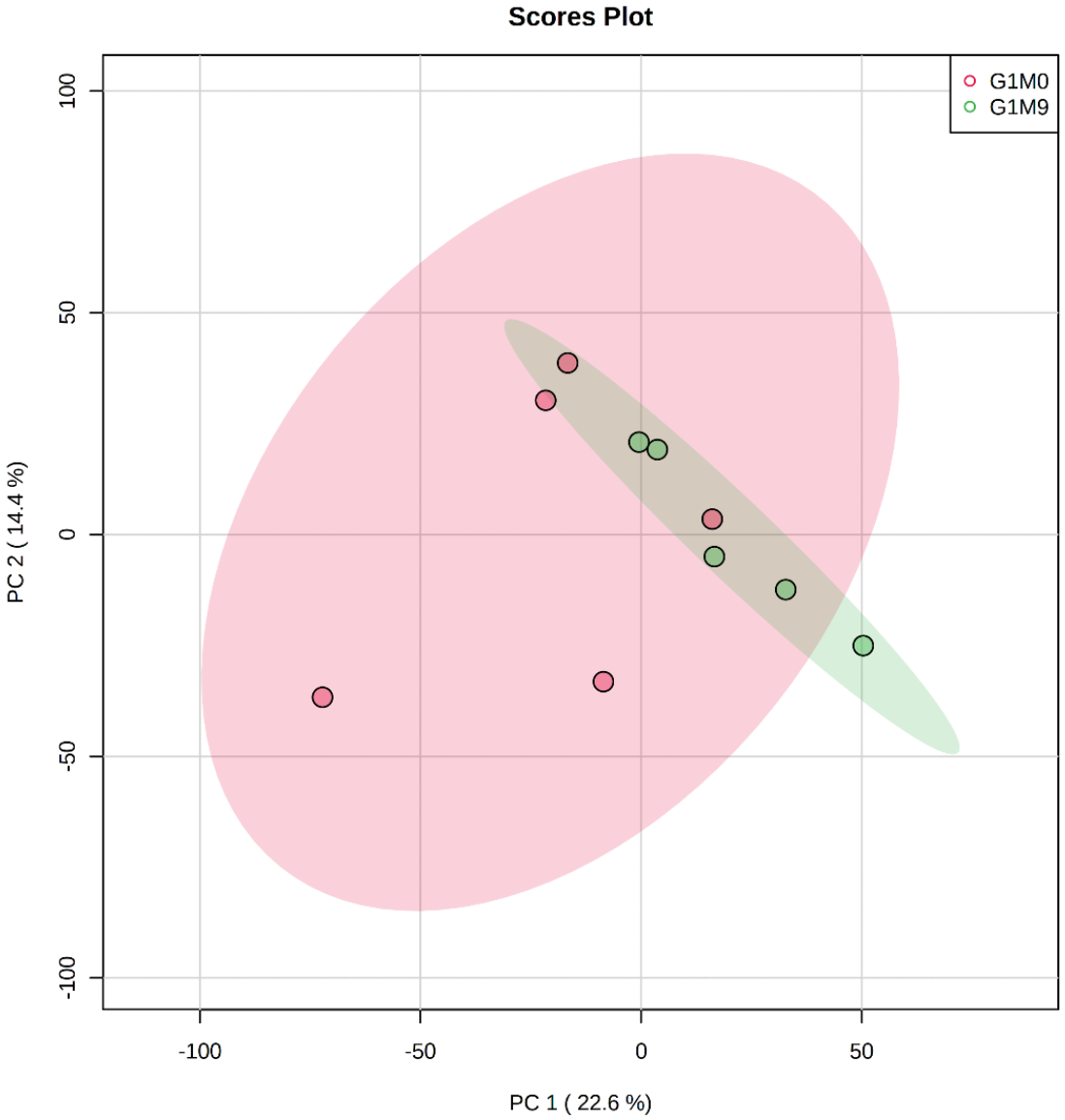
Source: Prepared by the authors

Supplementary Figure 9 - Variable Importance in Projection (VIP) of important features identified by PLS-DA. Scores plot with the comparison between the data from the first birth group (G1) in different postnatal moments, immediately after birth (M0) and with 3 days (M3), the explained variances are shown in brackets.



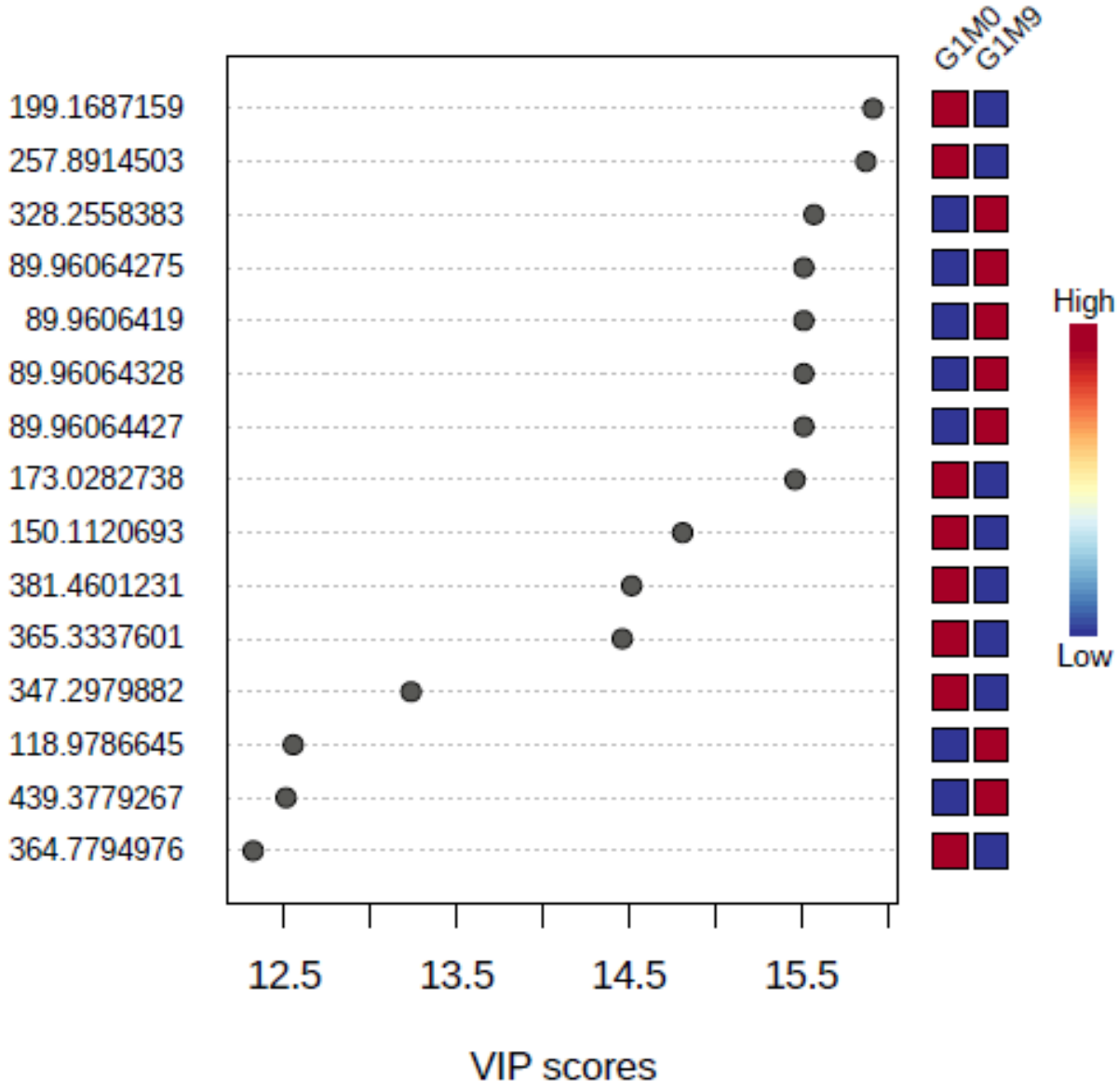
Source: Prepared by the authors

Supplementary Figure 10 - PCA scores plot with the comparison between the data from the first birth group (G1) in different postnatal moments, immediately after birth (M0) and with 9 days (M9), the explained variances are shown in brackets.



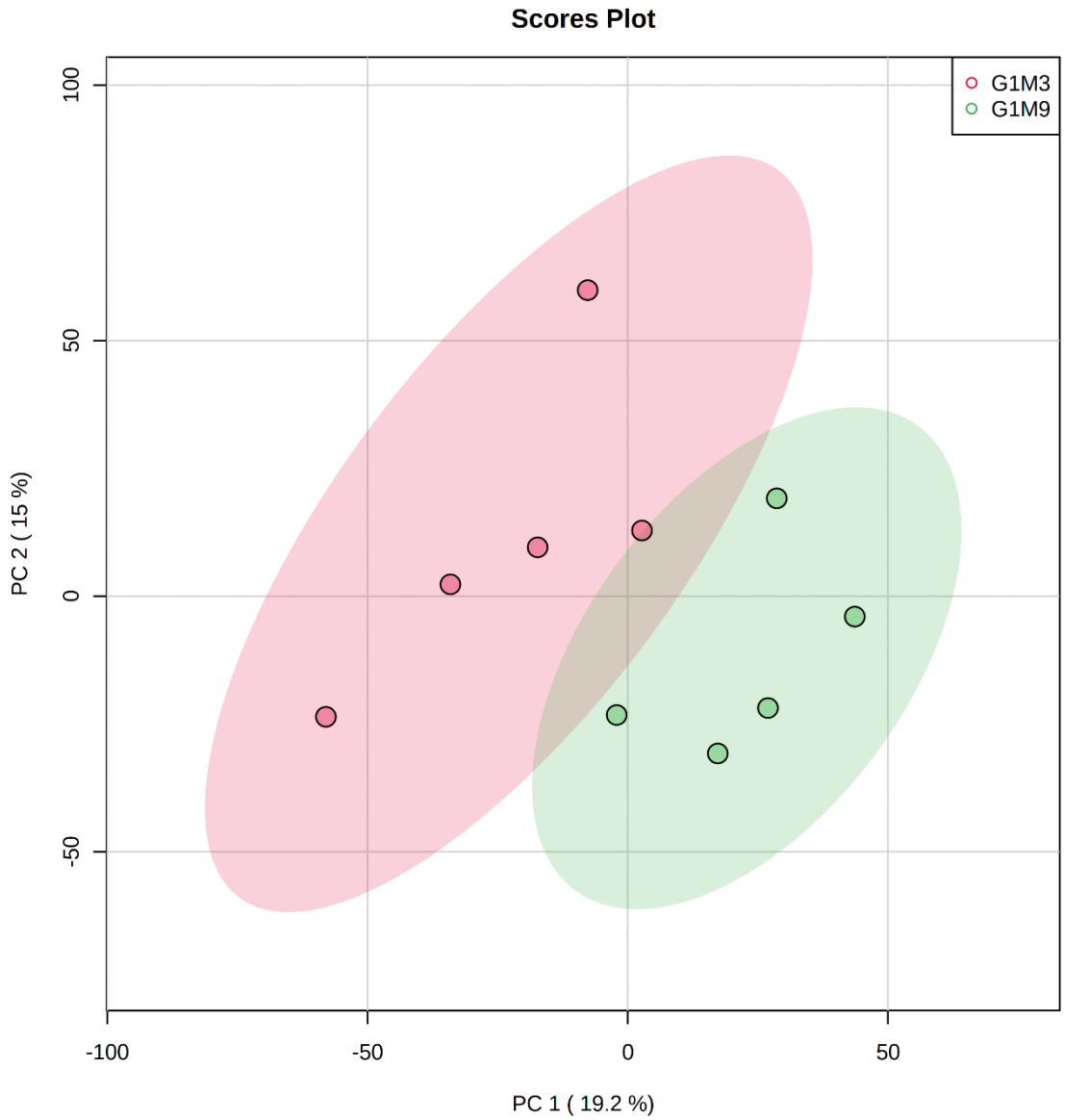
Source: Prepared by the authors

Supplementary Figure 11 - Variable Importance in Projection (VIP) of important features identified by PLS-DA. Scores plot with the comparison between the data from the first birth group (G1) in different postnatal moments, immediately after birth (M0) and with 9 days (M9), the explained variances are shown in brackets.



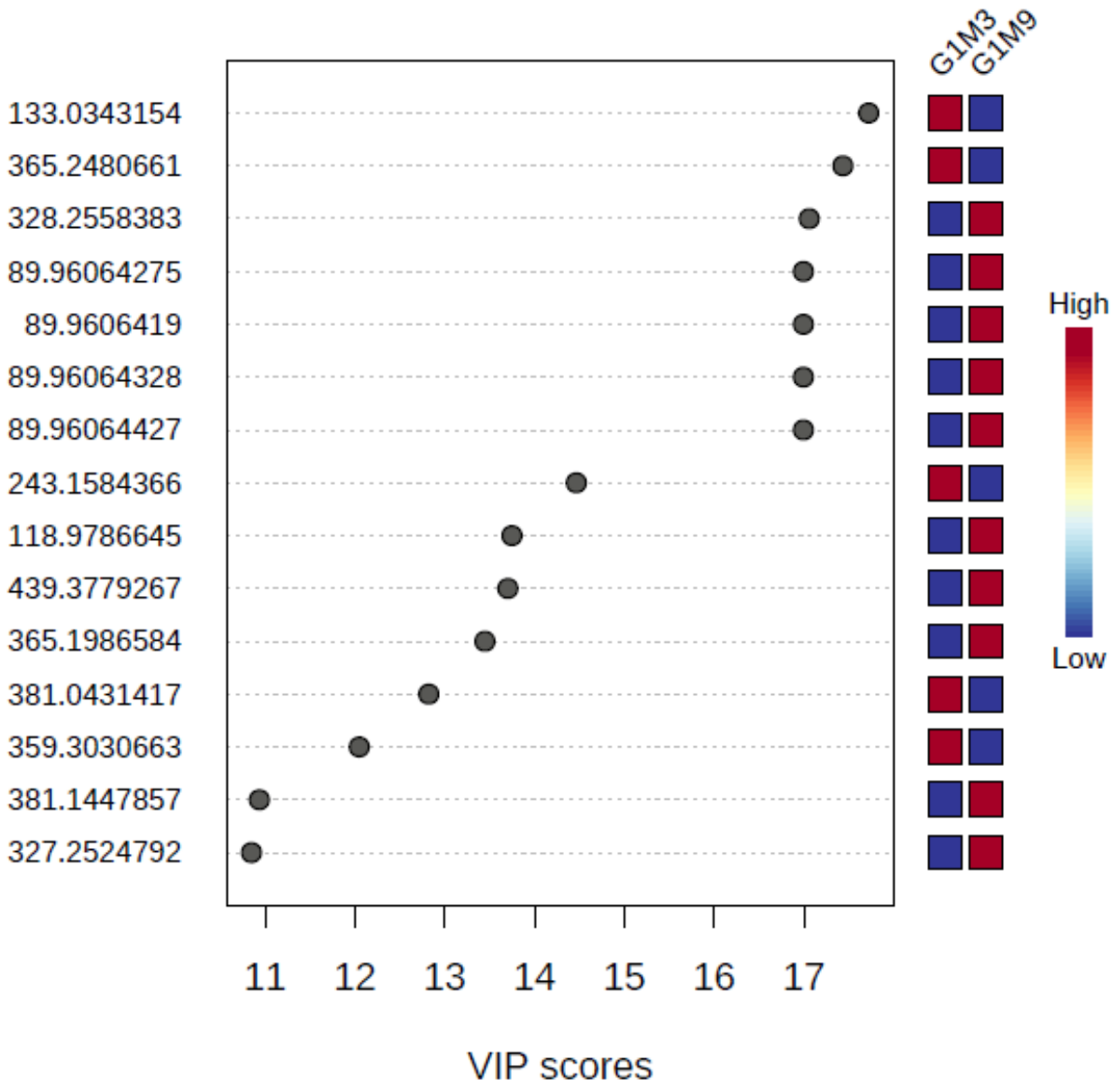
Source: Prepared by the authors

Supplementary Figure 12 - PCA scores plot with the comparison between the data from the first birth group (G1) in different postnatal moments, with 3 days (M3) and with 9 days (M9), the explained variances are shown in brackets.



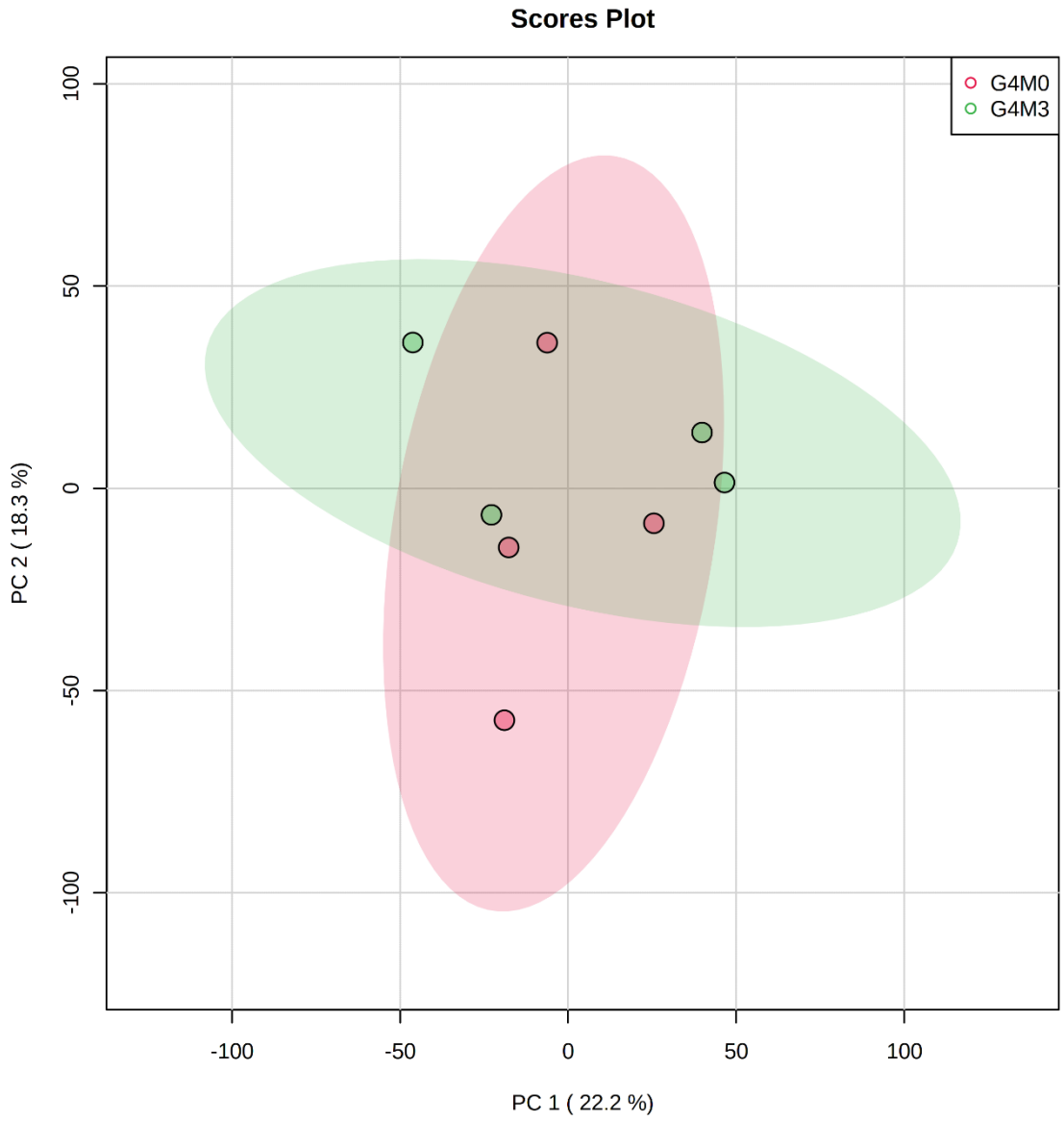
Source: Prepared by the authors

Supplementary Figure 13 - Variable Importance in Projection (VIP) of important features identified by PLS-DA. Scores plot with the comparison between the data from the first birth group (G1) in different postnatal moments, with 3 days (M3) and with 9 days (M9), the explained variances are shown in brackets.



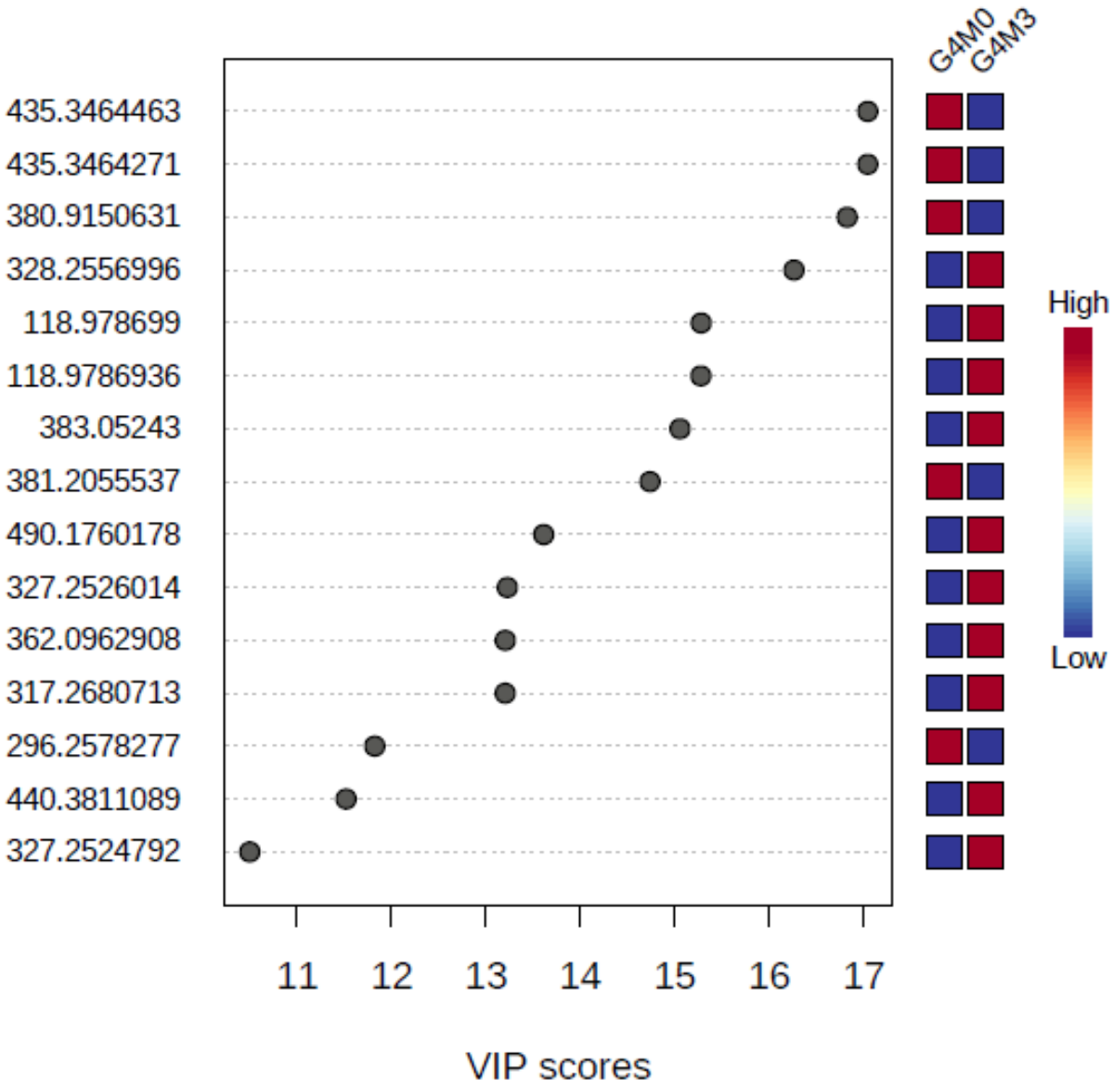
Source: Prepared by the authors

Supplementary Figure 14 - PCA scores plot with the comparison between the data from the CAEV negative animals with more than three births (G4) in different postnatal moments, immediately after birth (M0) and with 3 days (M3), the explained variances are shown in brackets.



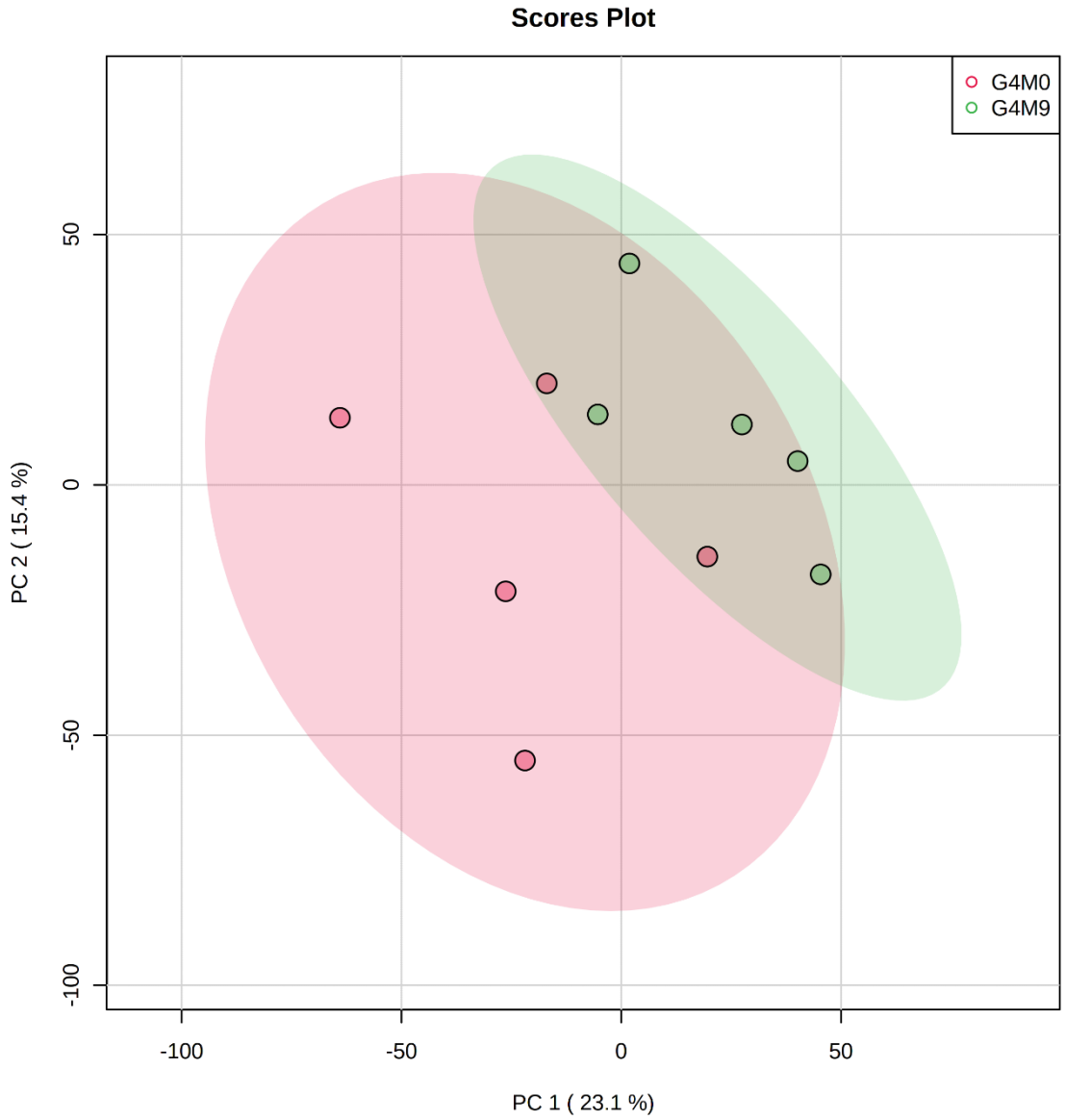
Source: Prepared by the authors

Supplementary Figure 15 - Variable Importance in Projection (VIP) of important features identified by PLS-DA. Scores plot with the comparison between the data from the CAEV negative animals with more than three births (G4) in different postnatal moments, immediately after birth (M0) and with 3 days (M3), the explained variances are shown in brackets.



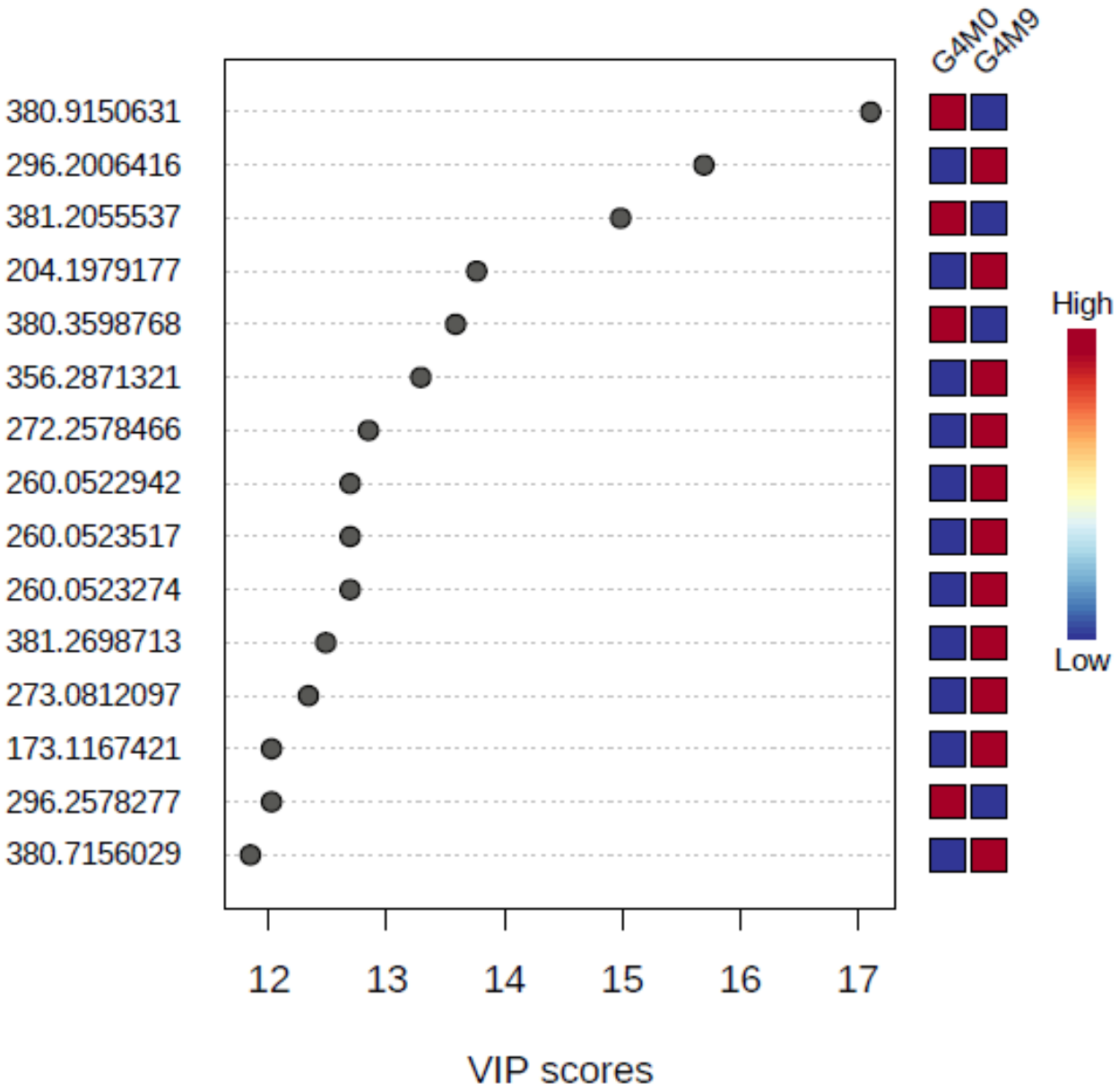
Source: Prepared by the authors

Supplementary Figure 16 - PCA scores plot with the comparison between the data from the CAEV negative animals with more than three births (G4) in different postnatal moments, immediately after birth (M0) and with 9 days (M9), the explained variances are shown in brackets.



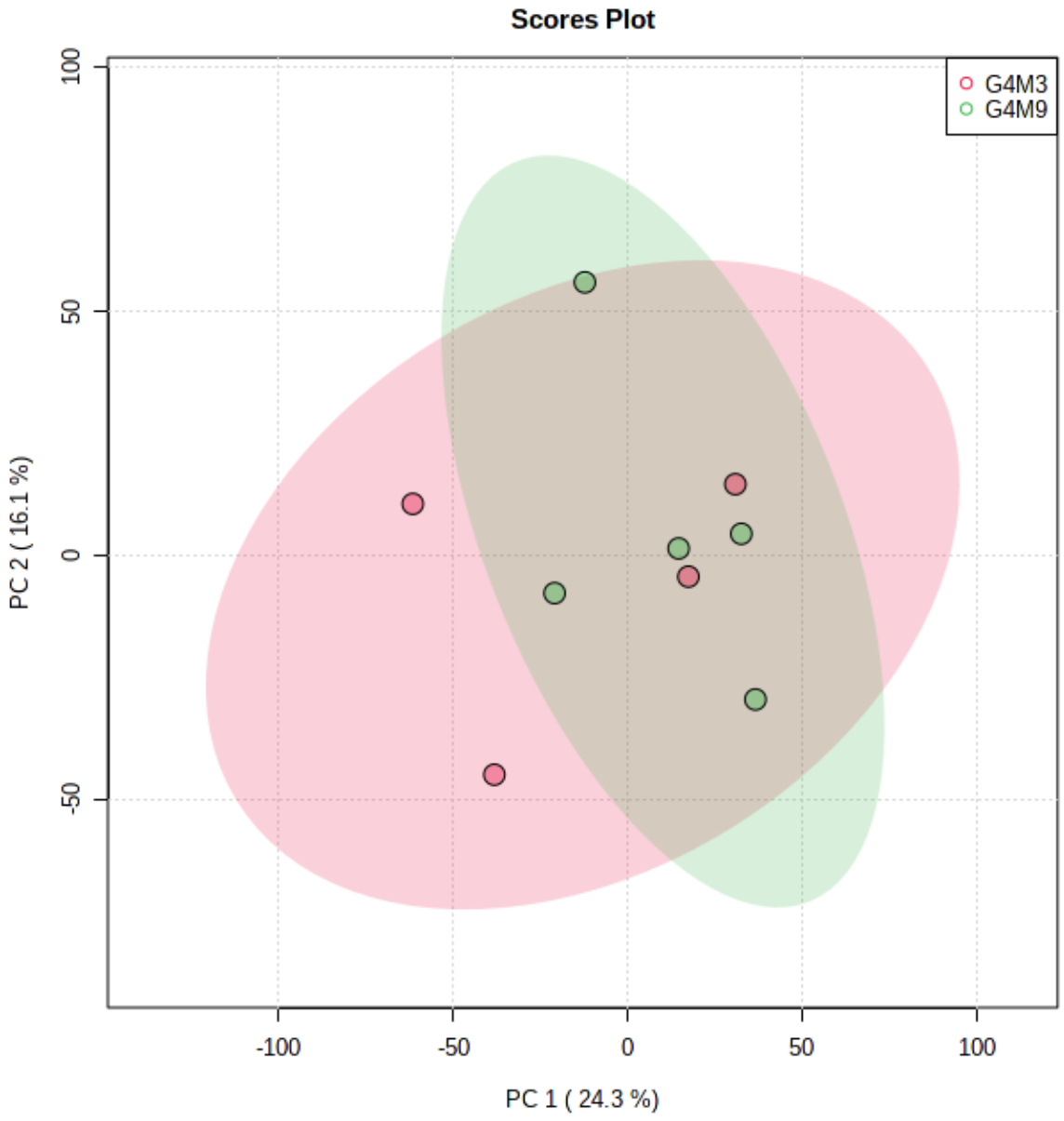
Source: Prepared by the authors

Supplementary Figure 17 - Variable Importance in Projection (VIP) of important features identified by PLS-DA. Scores plot with the comparison between the data from the CAEV negative animals with more than three births (G4) in different postnatal moments, immediately after birth (M0) and with 9 days (M9), the explained variances are shown in brackets.



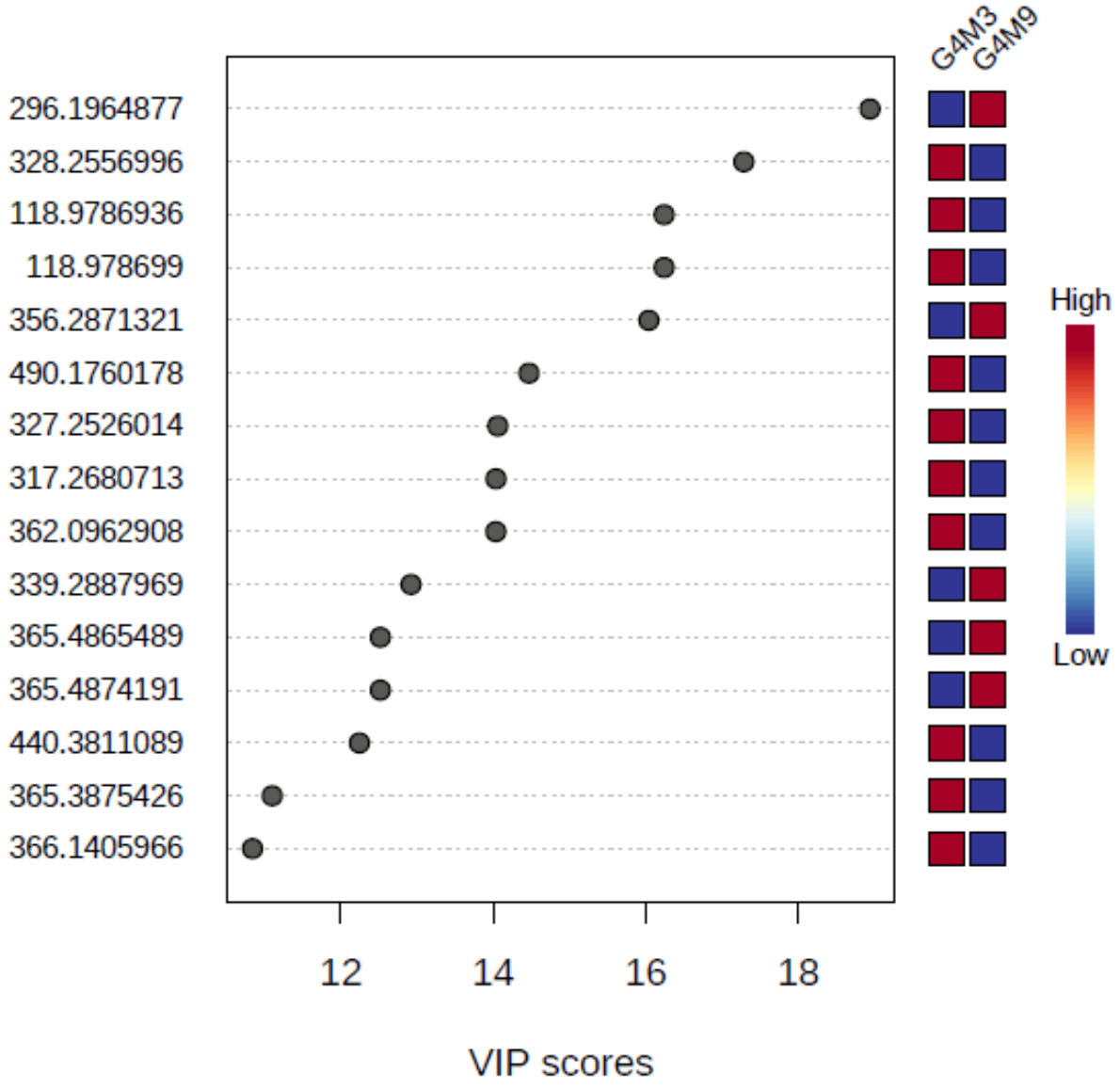
Source: Prepared by the authors

Supplementary Figure 18 - PCA scores plot with the comparison between the data from the CAEV negative animals with more than three births (G4) in different postnatal moments, with 3 days (M3) and with 9 days (M9), the explained variances are shown in brackets.



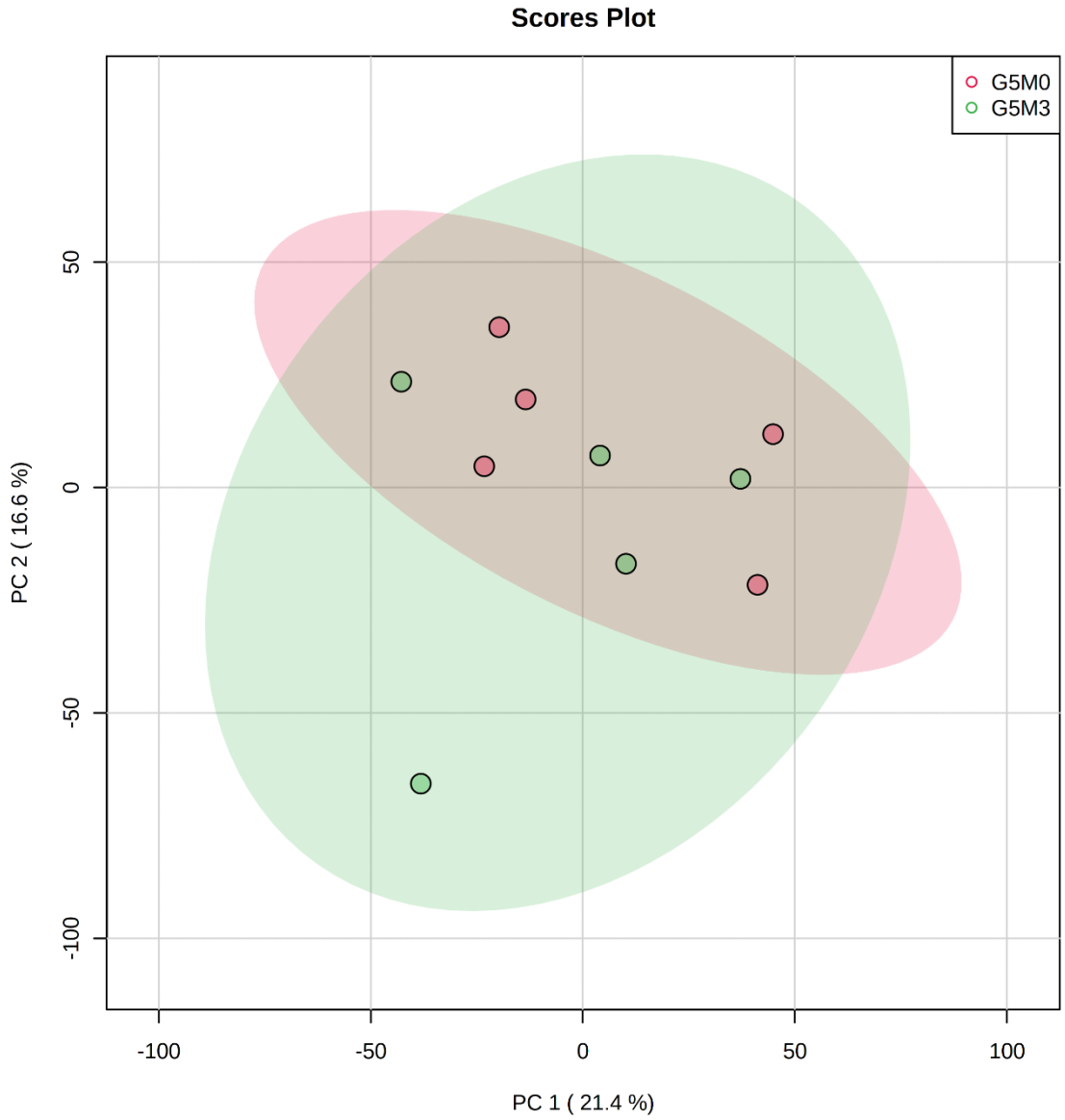
Source: Prepared by the authors

Supplementary Figure 19 - Variable Importance in Projection (VIP) of important features identified by PLS-DA. Scores plot with the comparison between the data from the CAEV negative animals with more than three births (G4) in different postnatal moments with 3 days (M3) and with 9 days (M9), the explained variances are shown in brackets.



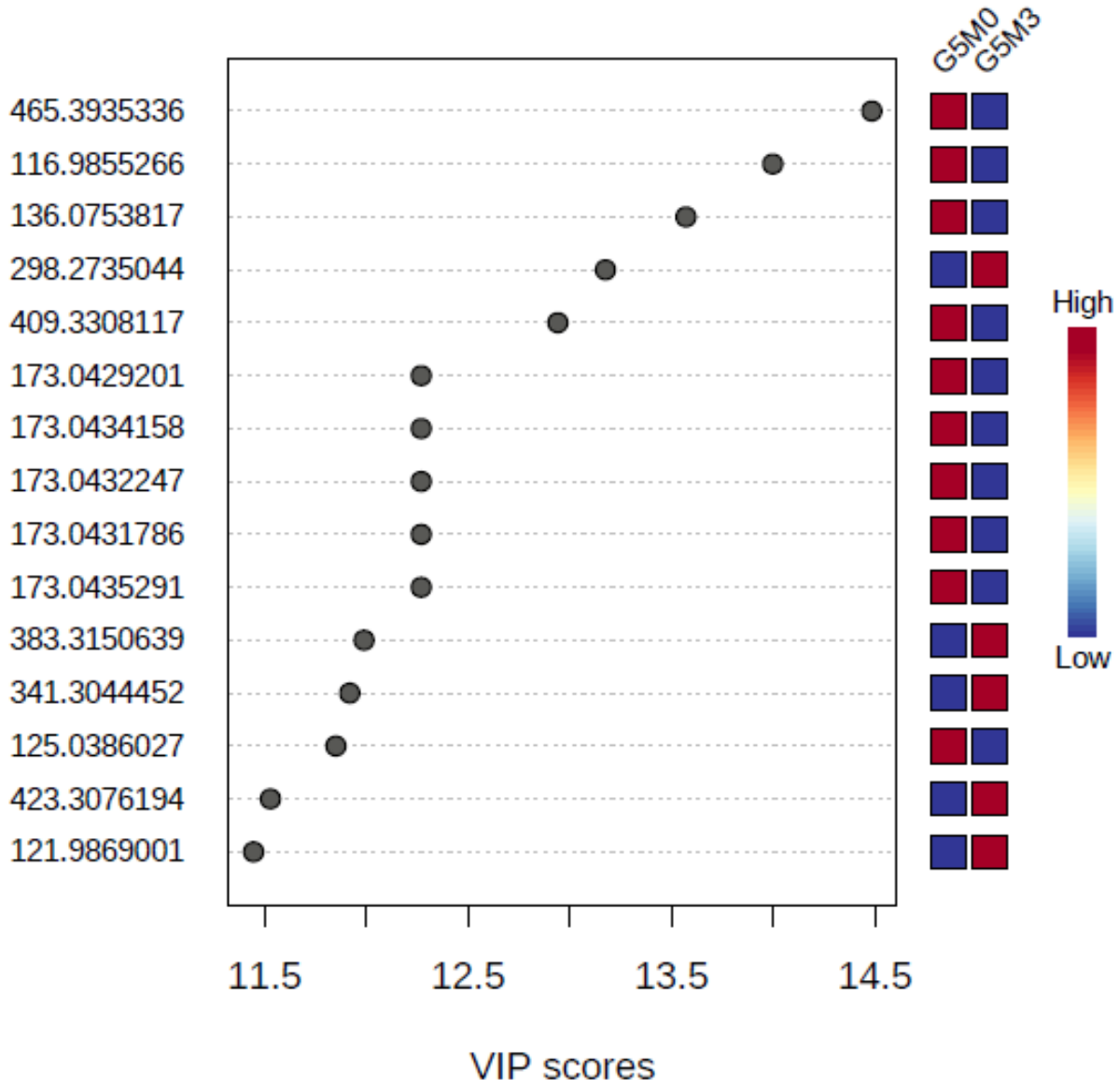
Source: Prepared by the authors

Supplementary Figure 20 - PCA scores plot with the comparison between the data from the CAEV positive animals with more than three births (G5) in different postnatal moments, immediately after birth (M0) and with 3 days (M3), the explained variances are shown in brackets.



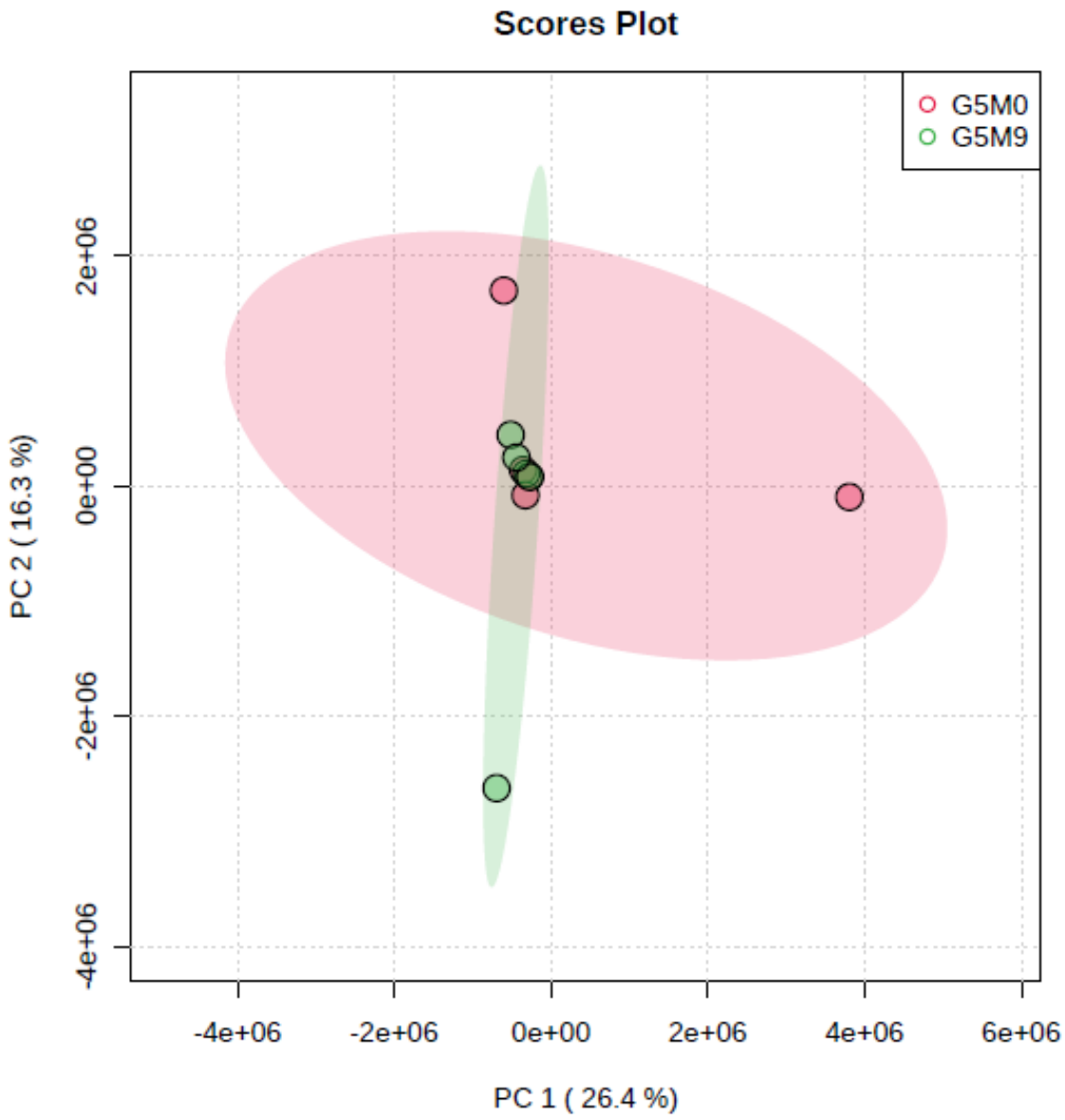
Source: Prepared by the authors

Supplementary Figure 21 - Variable Importance in Projection (VIP) of important features identified by PLS-DA. Scores plot with the comparison between the data from the CAEV positive animals with more than three births (G5) in different postnatal moments, immediately after birth (M0) and with 3 days (M3), the explained variances are shown in brackets.



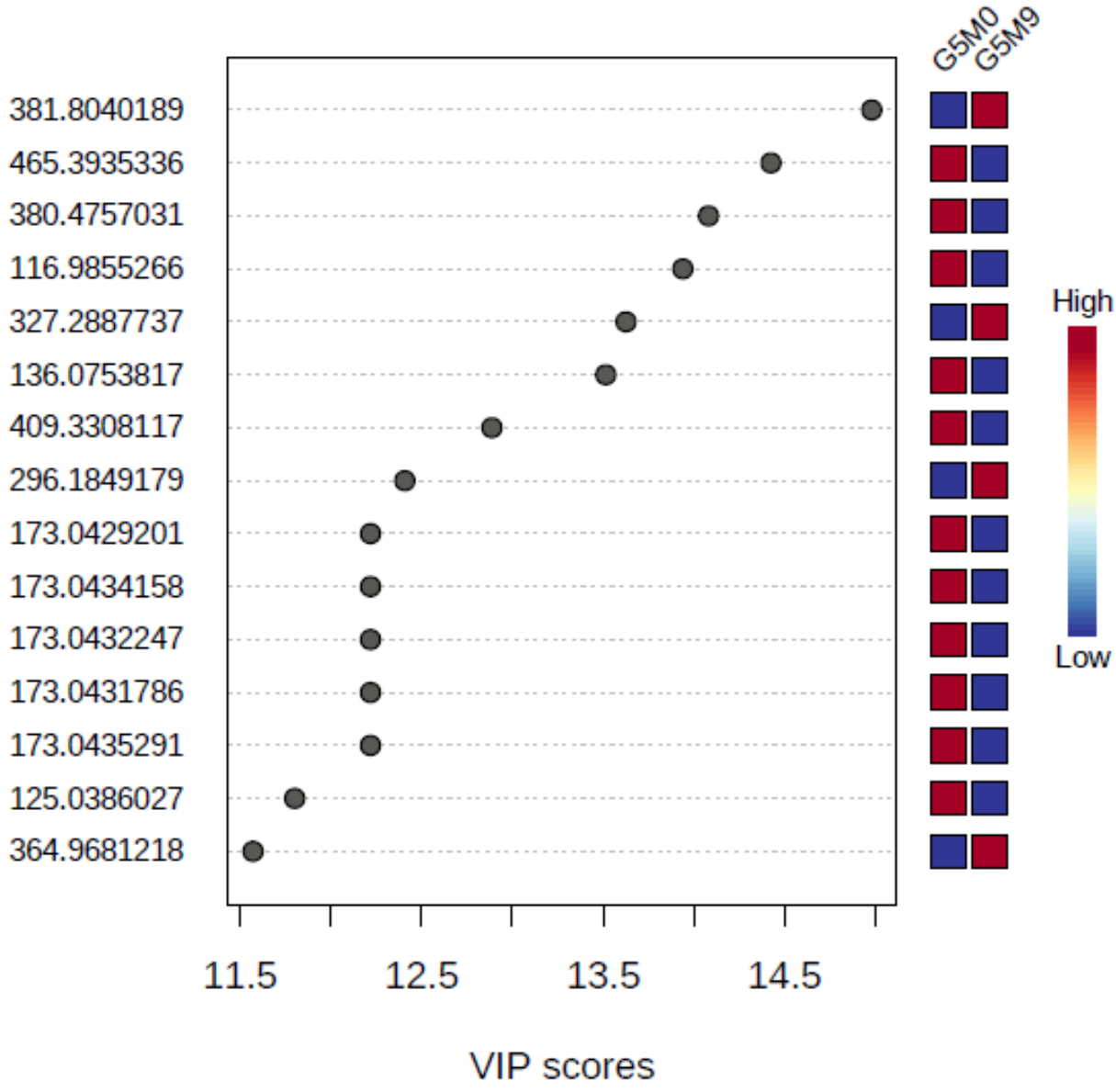
Source: Prepared by the authors

Supplementary Figure 22 - PCA scores plot with the comparison between the data from the CAEV positive animals with more than three births (G5) in different postnatal moments, immediately after birth (M0) and with 9 days (M9), the explained variances are shown in brackets.



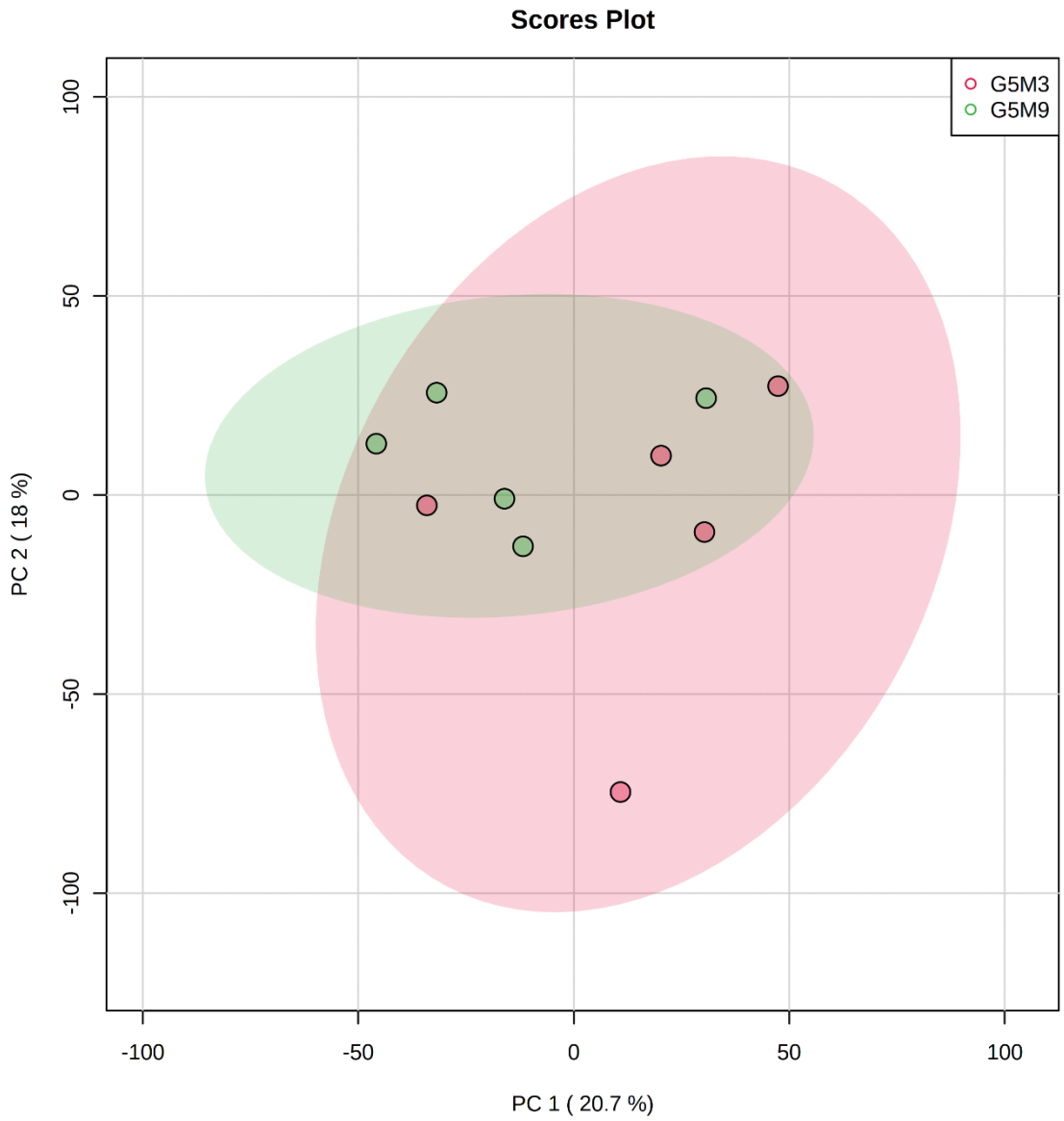
Source: Prepared by the authors

Supplementary Figure 23 - Variable Importance in Projection (VIP) of important features identified by PLS-DA. Scores plot with the comparison between the data from the CAEV positive animals with more than three births (G5) in different postnatal moments, immediately after birth (M0) and with 9 days (M9), the explained variances are shown in brackets.



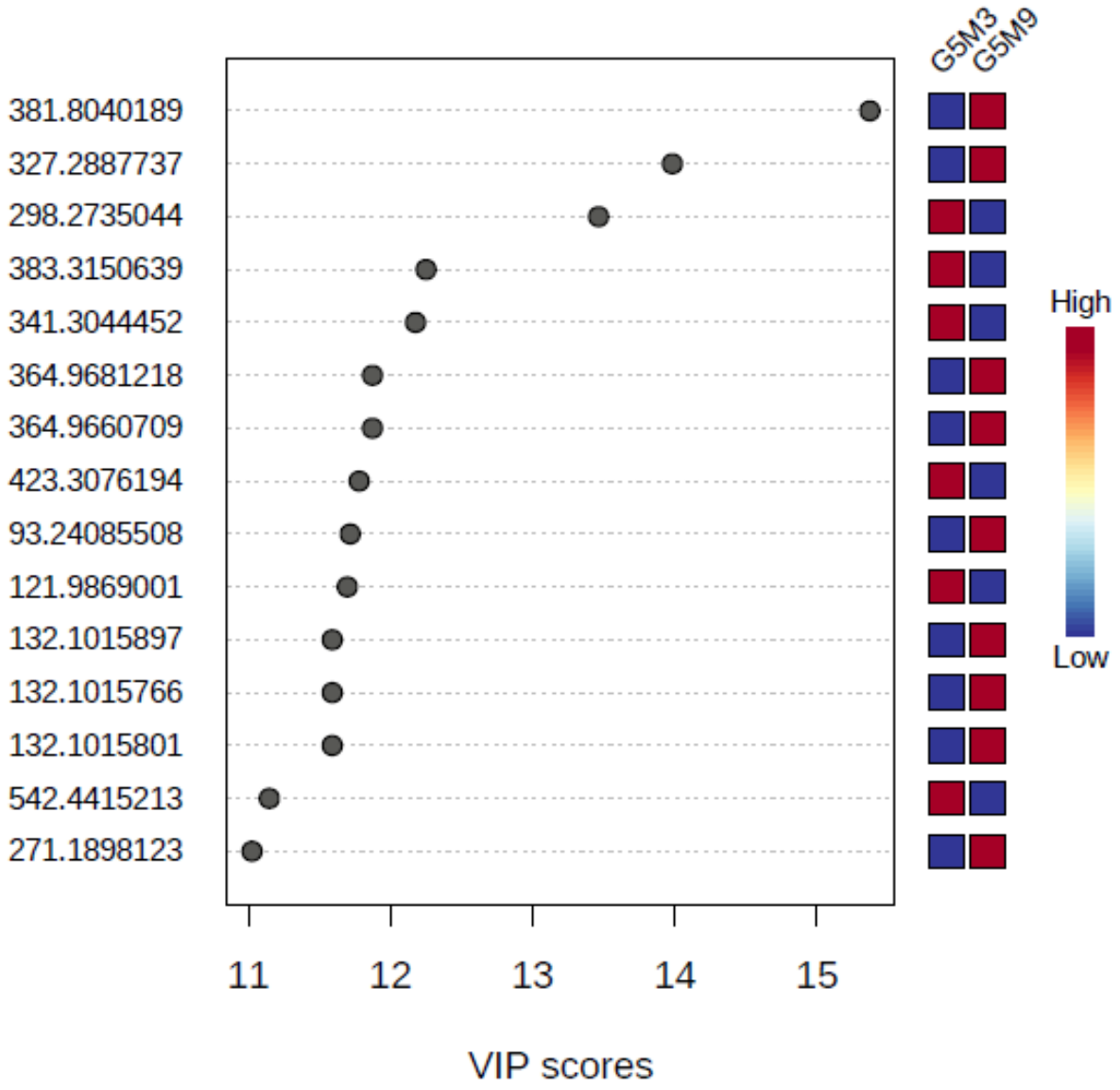
Source: Prepared by the authors

Supplementary Figure 24 - PCA scores plot with the comparison between the data from the CAEV positive animals with more than three births (G5) in different postnatal moments, with 3 days (M3) and with 9 days (M9), the explained variances are shown in brackets.



Source: Prepared by the authors

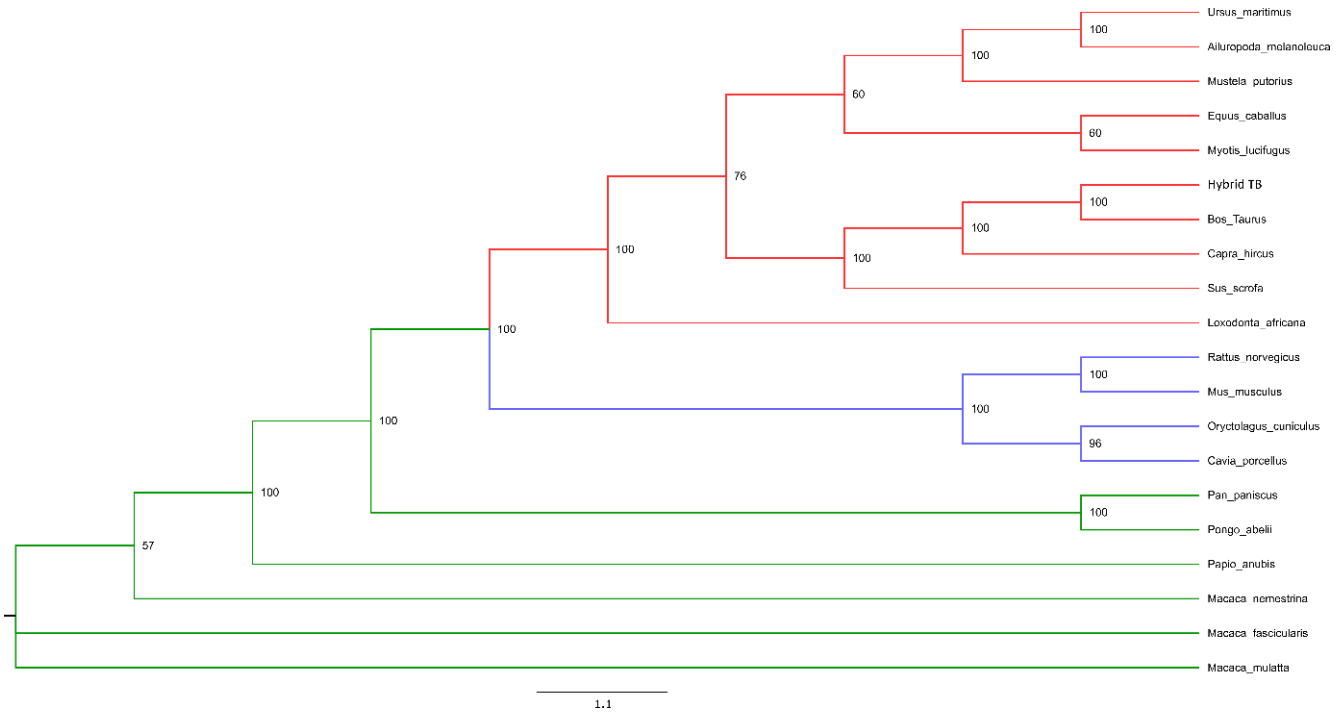
Supplementary Figure 25 - Variable Importance in Projection (VIP) of important features identified by PLS-DA. Scores plot with the comparison between the data from the CAEV positive animals with more than three births (G5) in different postnatal moments with 3 days (M3) and with 9 days (M9), the explained variances are shown in brackets.



Source: Prepared by the authors

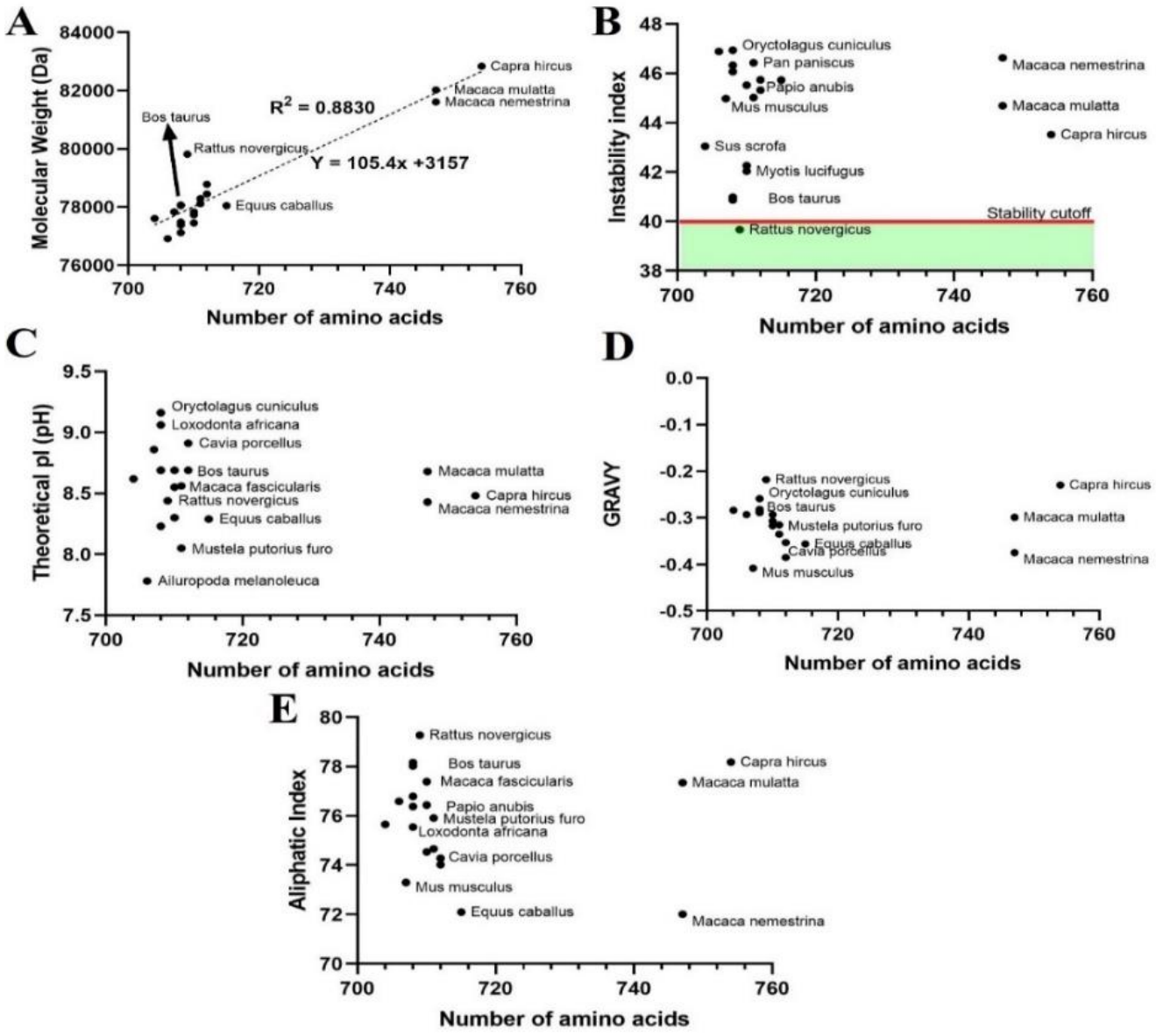
APÊNDICE D - Arquivo Suplementar ao Capítulo 3

Supplementary Figure 1 - Phylogenetic tree of nucleotides sequences of 20 lactotransferrin of different species of mammals. The lines in their respective colors demonstrate: Clade A as red, Clade B as blue and Clade C as green. Hybrid TB means one animal produced with the reproduction from two species Bos taurus and Bos indicus.



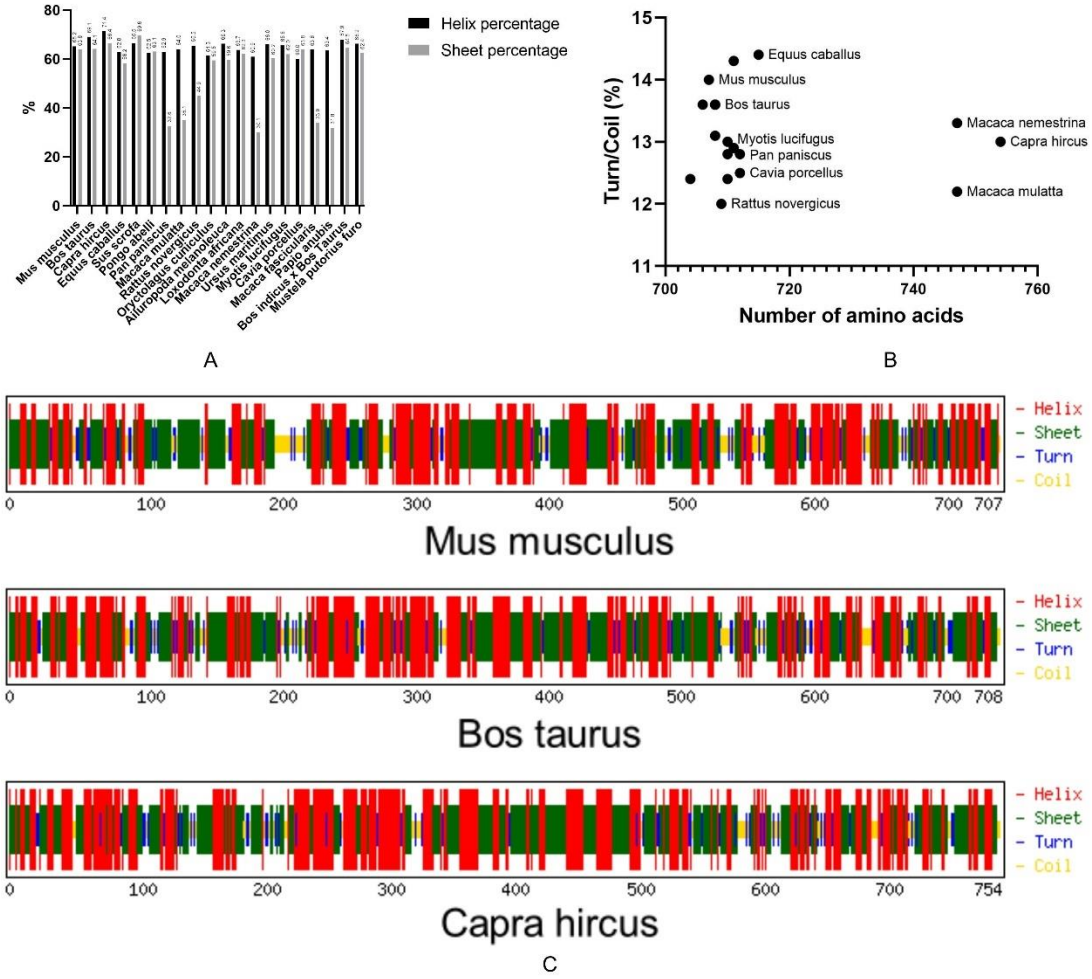
Source: Prepared by the authors

Supplementary Figure 2 - Physicochemical properties of lactotransferrin of 20 different species of mammals. Legend: For the Instability index (2B), values equal or below 40 show more stable proteins.



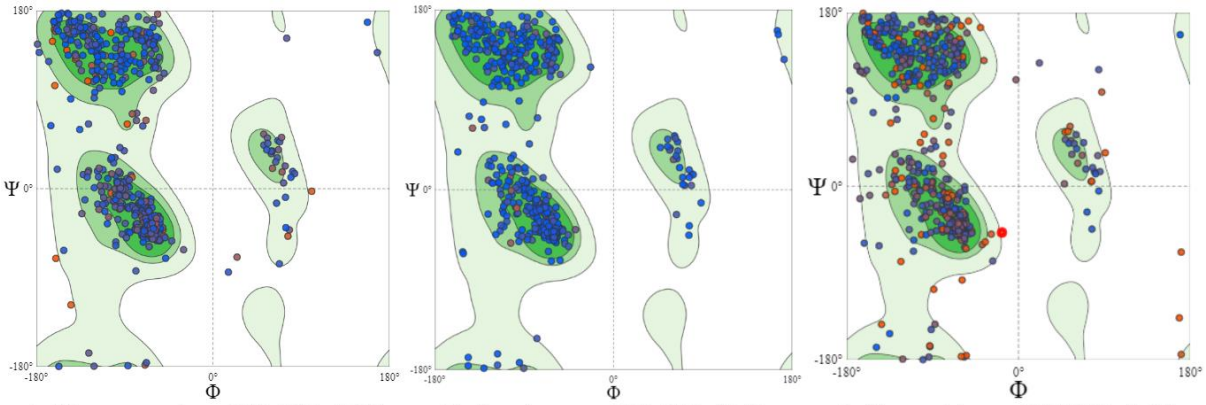
Source: Prepared by the authors

Supplementary Figure 3 - Representation of helixes, sheets and turns of lactotransferrin of *Mus musculus, Bos taurus* and *Capra hircus* amino acids data.



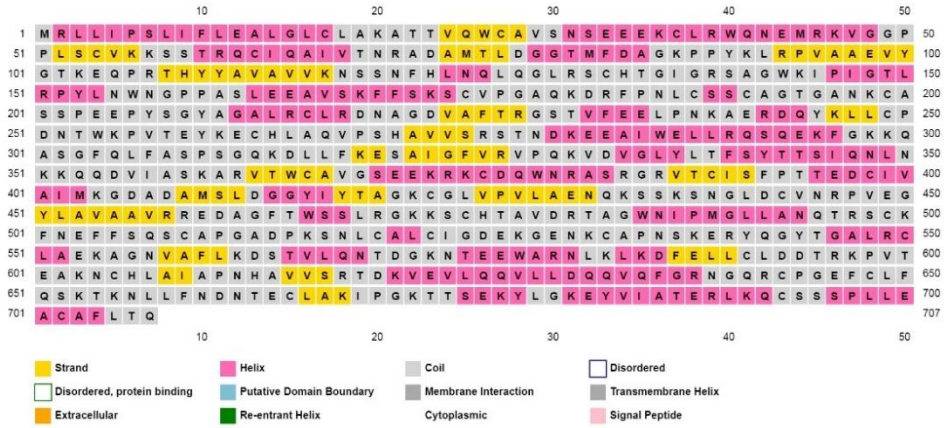
Source: Prepared by the authors

Supplementary Figure 4 - Ramachandran analysis of lactotransferrin in 20 species of mammalians, exemplified for *Mus musculus, Bos taurus* and *Capra hircus*. Alpha helix and beta sheet (Left) over 80% and turn on the right. To colors are related with specific regions on the model related with the Supplementary Figure 8.

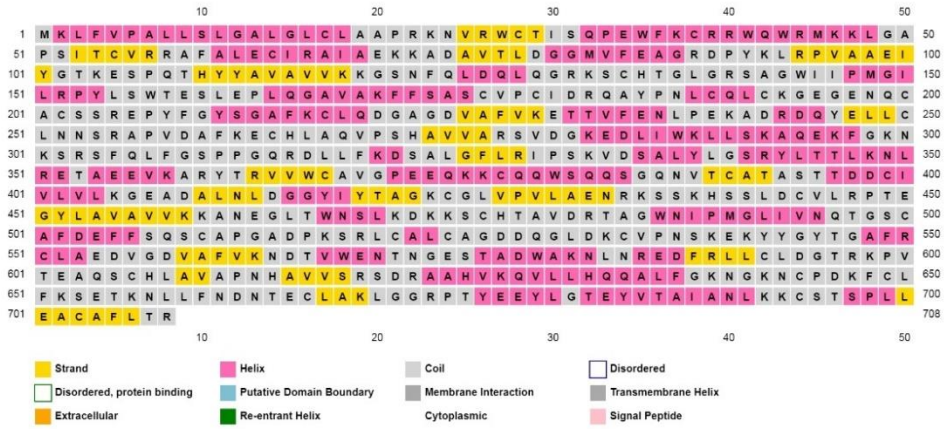


Source: Prepared by the authors

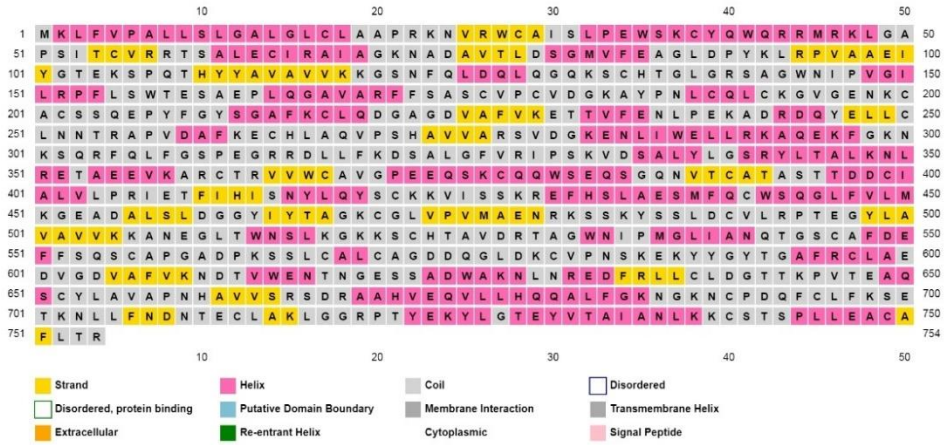
Supplementary Figure 5 - Strand, Helix, Coil and structural aspects of lactotransferrin in *Mus musculus, Bos taurus* and *Capra hircus.* Below in the Figure its possible to see the colors meaning and identification.



A, Mus musculus



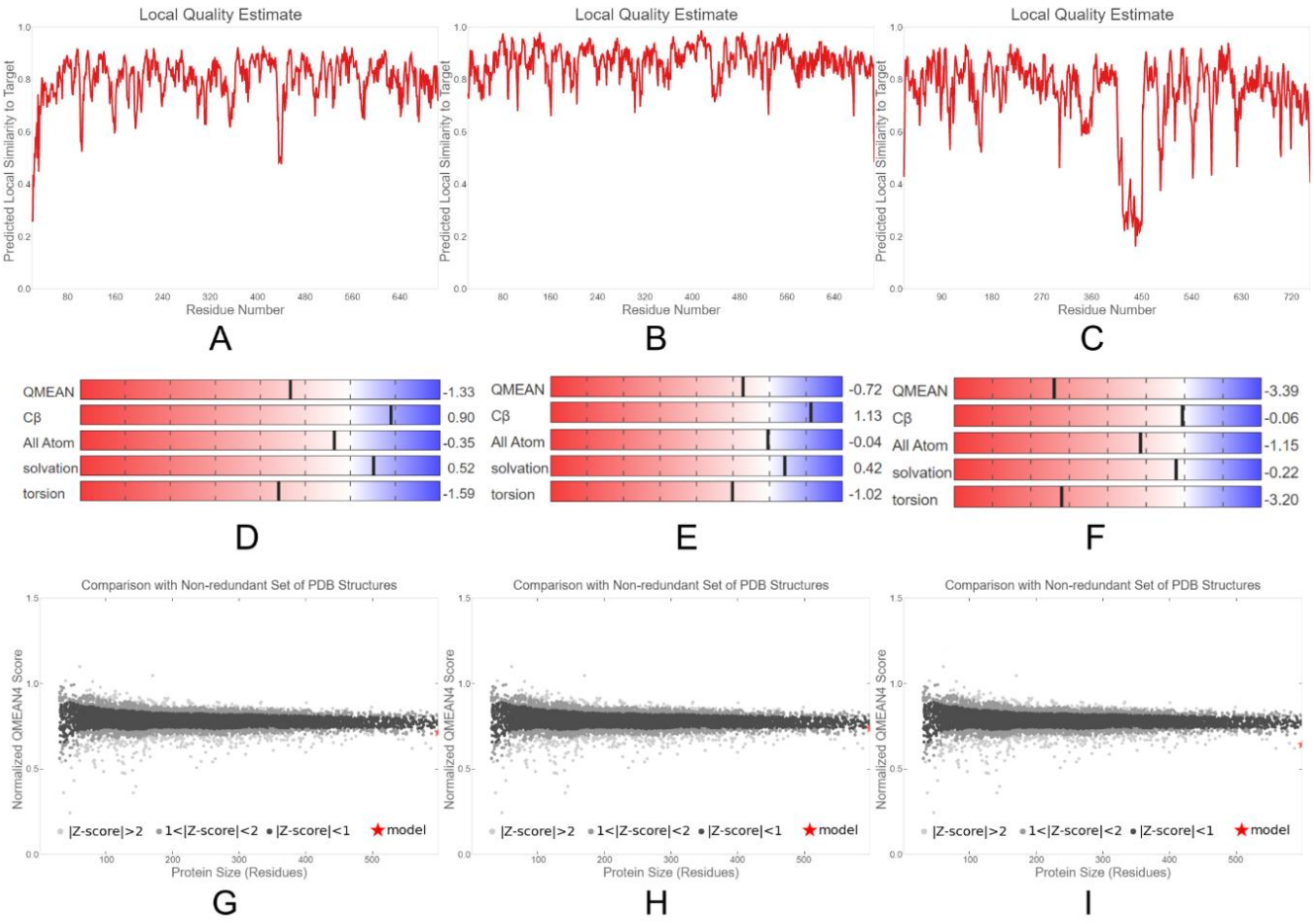
B, Bos taurus



C, Capra hircus

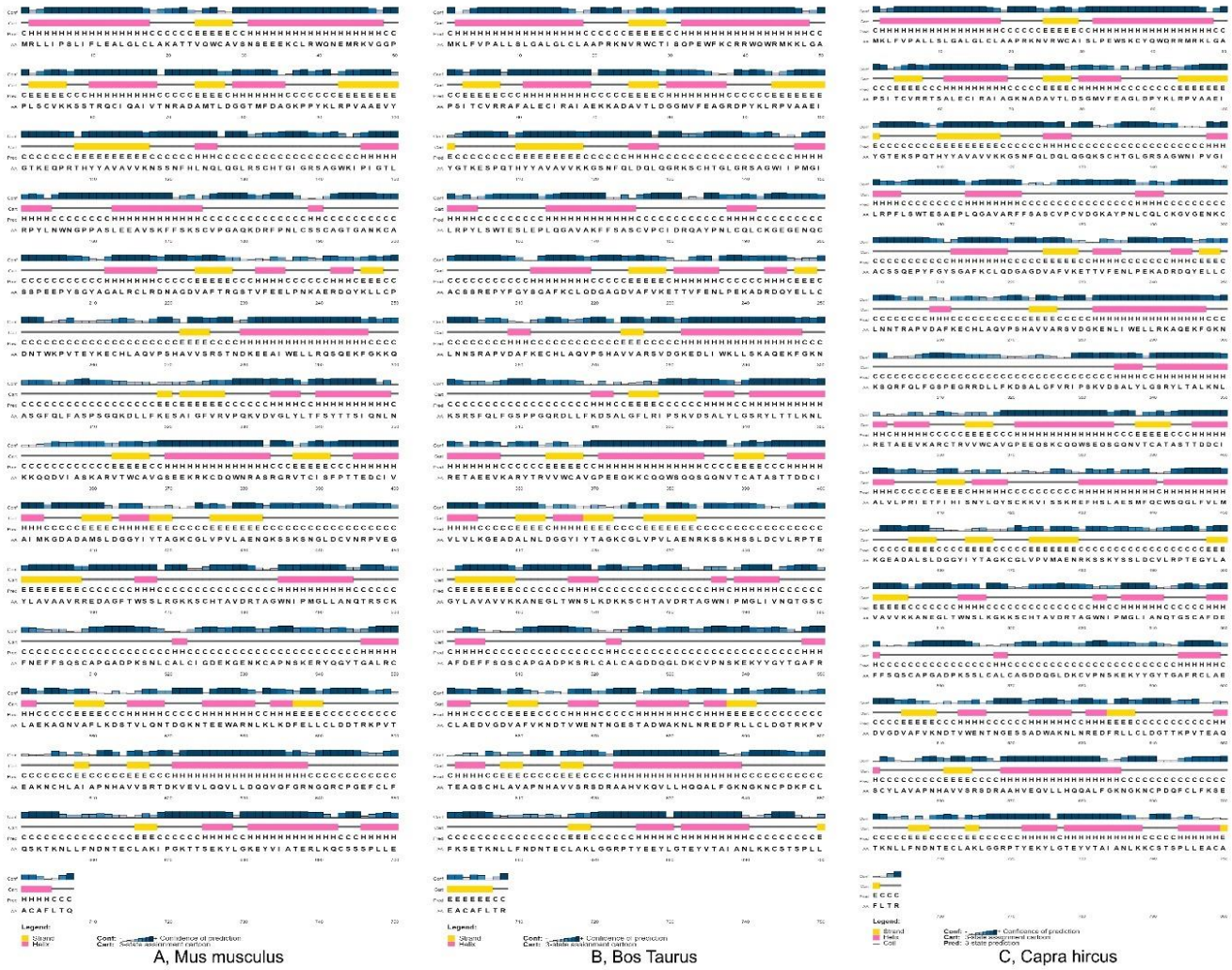
Source: Prepared by the authors

Supplementary Figure 6 - Evaluation of model stability using PSIPRED 4.0 (Predict Secondary Structure) and QMEAN value as reference of how trustable it is the model.



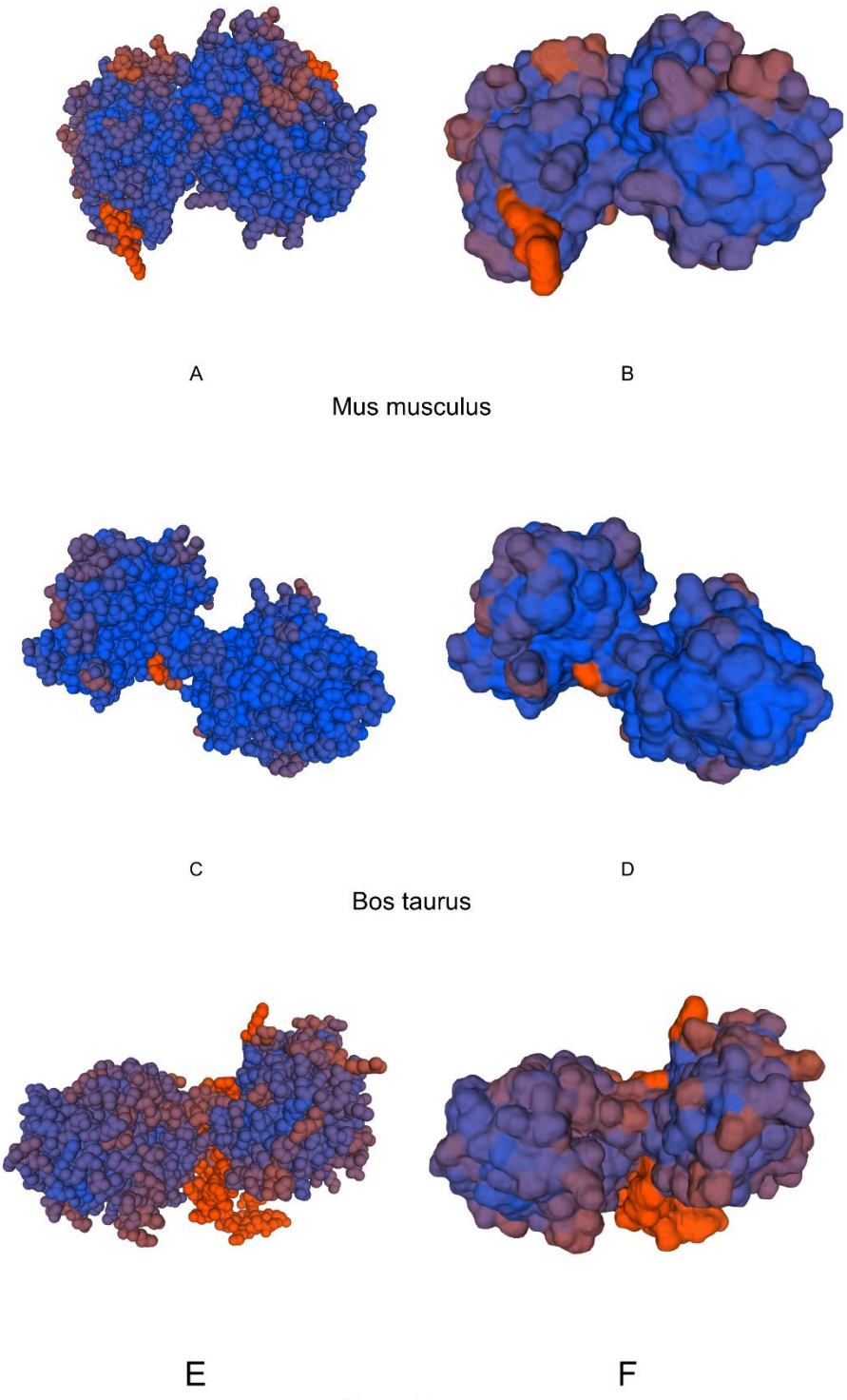
Source: Prepared by the authors

Supplementary Figure 7 - Model stability by the evaluation of the structural aspects from PSIPRED the confidence it`s expressed by the blue squares. Below in the Figure its possible to see the colours meaning and identification.



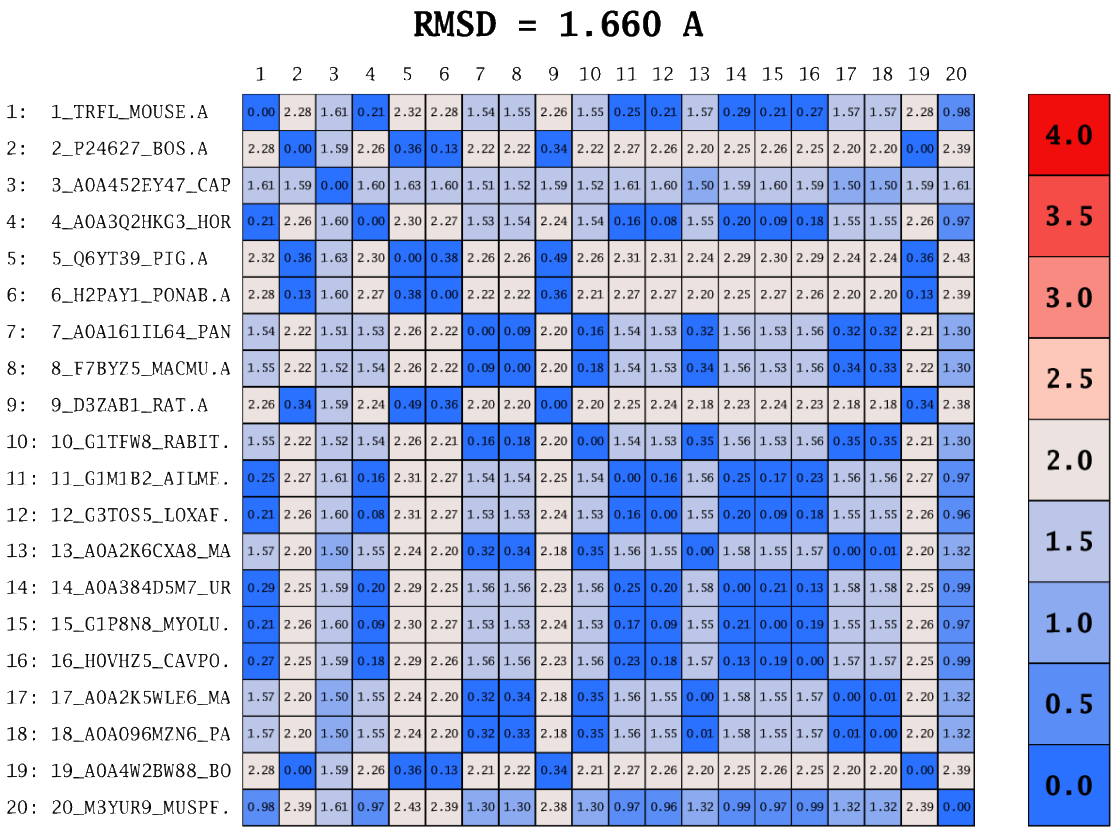
Source: Prepared by the authors

Supplementary Figure 8 - Spacefill and Surface model from *Mus musculus*, *Bos taurus* and *Capra hircus* data obtained by SWISSMODEL and selected with the best QMEAN. The colors are related with Ramachandran plots in the Supplementary Figure 4.



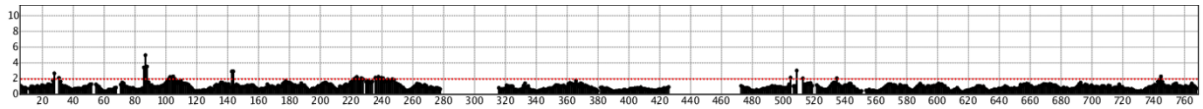
Source: Prepared by the authors

Supplementary Figure 09 - RMSD (Root Mean Square Deviation) matrix for structural analysis of models.



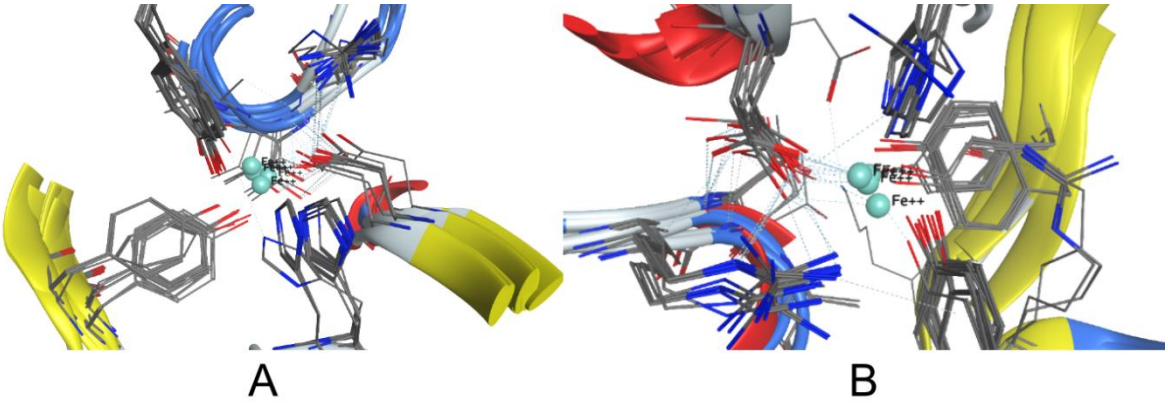
Source: Prepared by the authors

Supplementary Figure 10 - Average RMSD (Root Mean Square Deviation) value verses Amino Acid position of the *in silico* digestion.



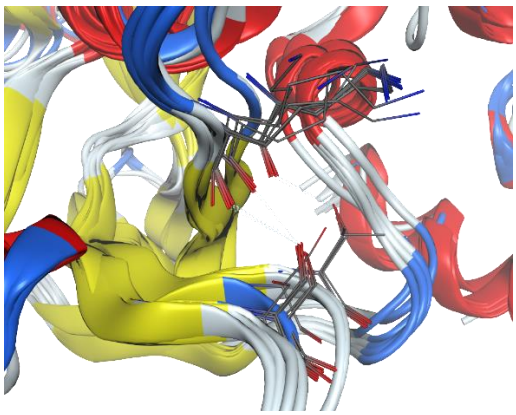
Source: Prepared by the authors

Supplementary Figure 11 - Iron regions marked with the Fe^{++}, from the model comparison, related with the Figure 3 in the main paper.



Source: Prepared by the authors

Supplementary Figure 12 - The conserved proposed Serine Protease active site across all species, with the Lysine on top and Serine on the bottom.



Source: Prepared by the authors

Supplementary Figure 13 - A: Amino acid alignment based on structural alignment, showing the extension of the *Capra hircus*. (1_TRFL = *Mus musculus*, 2_P24627 = *Bos taurus* and 3_A0A452 = *Capra hircus*). B: FASTA sequence of the 46 AA loop region.

```
1_TRFL_M NRASRGRVTCISFPTTEDCIVAIM.....................................
2_P24627 SQQSGQNVTCATASTTDDCIVLVL.....................................
3_A0A452 SEQSGQNVTCATASTTDDCIALVLPRIETFIHISNYLQYSCKKVISSKREFHSLAESMFQ

1_TRFL_M ..........KGDADAMSLDGGYIYTAGKCGLVPVLAENQKSSKSNGLDCVNRPVEGYLA
2_P24627 ..........KGEADALNLDGGYIYTAGKCGLVPVLAENRKSSKHSSLDCVLRPTEGYLA
3_A0A452 CWSQGLFVLMKGEADALSLDGGYIYTAGKCGLVPVMAENRKSSKYSSLDCVLRPTEGYLA
```

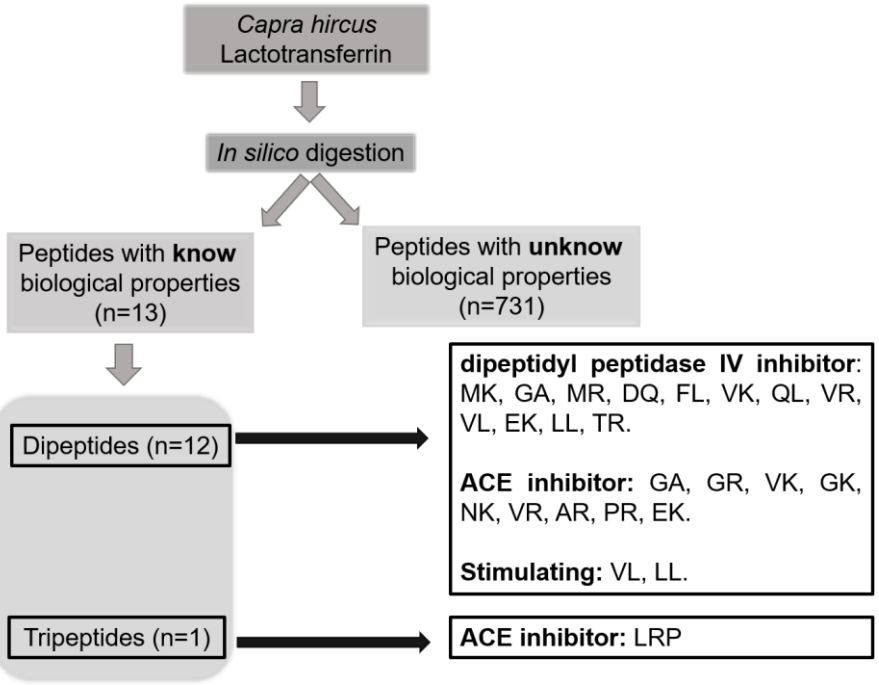
A

```
>3_A0A452EY47_CAPHI.A
PRIETFIHISNYLQYSCKKVISSKREFHSLAESMFQCWSQGLFVLM
```

B

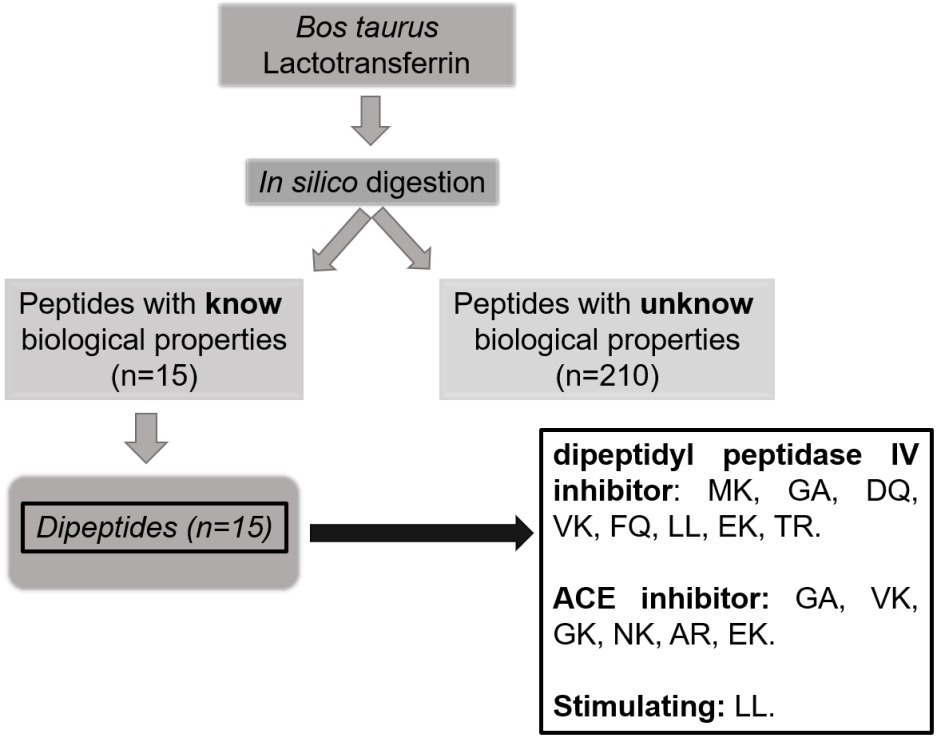
Source: Prepared by the authors

Supplementary Figure 15 - Sequences and functional properties of peptides derived from *in-silico* digestion of *Capra hircus* lactoferrin. ACE-I: Angiotensin converting enzyme inhibitor; DPP: dipeptidyl peptidase; R: Arginine; N: Asparagine; D: Aspartic acid; C: Cysteine; E: Glutamic acid; Q: Glutamine; G: Glycine; H: Histidine; I: Isoleucine; L: Leucine; K: Lysine; M: Methionine; F: Phenylalanine; P: Proline; S: Serine; T: Threonine; Y: Tyrosine; V: Valine.



Source: Prepared by the authors; Adaptation from Barati et al., 2020

Supplementary Figure 16 - Sequences and functional properties of peptides derived from *in-silico* digestion of *Bos taurus* lactoferrin. ACE-I: Angiotensin converting enzyme inhibitor; DPP: dipeptidyl peptidase; R: Arginine; N: Asparagine; D: Aspartic acid; C: Cysteine; E: Glutamic acid; Q: Glutamine; G: Glycine; H: Histidine; I: Isoleucine; L: Leucine; K: Lysine; M: Methionine; F: Phenylalanine; P: Proline; S: Serine; T: Threonine; Y: Tyrosine; V: Valine.



Source: Prepared by the authors; Adaptation from Barati et al., 2020

Supplementary Table 1 - Biological aspect of the models by I-TASSER using Consensus.

Consensus prediction of GO terms	Numbers	All Species
Molecular function	GO:0008199	ferric iron binding
	GO:0008201	heparin binding
	GO:0004857	enzyme inhibitor activity
	GO:0005515	protein binding
	GO:0004252	serine-type endopeptidase activity
Biological process	GO:0006879	cellular iron ion homeostasis
	GO:0006826	iron ion transport
	GO:0042742	defense response to bacterium
	GO:0043086	negative regulation of catalytic activity
	GO:0006959	humoral immune response
Cellular component	GO:0005576	extracellular region
	GO:0030141	secretory granule
	GO:0005769	early endosome
	GO:0005770	late endosome
	GO:0005905	clathrin-coated pit
	GO:0016324	apical plasma membrane
	GO:0055037	recycling endosome
	GO:0048471	perinuclear region of cytoplasm
	GO:0030139	endocytic vesicle
	GO:0009925	basal plasma membrane

Source: Prepared by the authors

Supplementary Table 2 - Identification of Cleavege sites, number of cleavages and activity of digested peptides in *Capra hircus, Bos taurus* and *Mus musculus* lactoferrin data.

Species	Cleavege sites	Number of Cleavages	Activity of digested peptides	Score
Capra hircus	2-3-8-9-10-11-13-14-15-16-17-18-22-23-26-32-37-43-44-46-47-57-58-61-62-66-71-77-78-83-84-87-92-105-118-119-122-123-124-125-127-128-132-138-140-150-153-155-163-170-171-183-188-190-191-193-199-209-214-215-216-217-226-227-229-233-234-237-240-243-247-248-249-250-251-255-260-261-262-265-266-277-282-285-288-289-290-291-292-296-297-299-301-304-305-307-308-314-315-318-319-320-323-324-325-326-328-332-336-337-338-339-342-343-346-347-348-349-351-358-360-363-376-401-402-404-406-409-410-416-417-422-423-428-429-433-438-439-445-446-447-448-449-451-456-457-458-459-469-480-481-484-487-488-491-498-499-505-506-510-511-515-516-517-519-520-528-537-538-547-548-550-551-552-563-566-568-569-576-577-579-585-587-594-595-596-597-606-607-609-627-628-631-634-635-636-638-639-653-654-665-668-675-676-677-681-682-683-685-688-693-694-695-696-697-698-702-703-705-706-712-713-715-716-724-725-736-737-738-739-744-746-750-751-752	235	ACE inhibitor;dipeptidyl peptidase IV inhibitor;stimulating	31
Bos taurus	2-3-8-9-10-11-13-14-15-16-17-18-22-23-26-35-36-37-39-40-44-46-47-57-58-61-62-66-71-72-77-78-83-84-88-92-104-118-119-122-	225	ACE inhibitor;dipeptidyl peptidase IV inhibitor;stimulating	

	123-124-125-127-128-131-132-138-140-150-155-160-163-170-171-182-188-190-191-193-205-209-214-215-216-217-226-227-229-233-234-237-240-243-247-248-249-250-251-255-260-261-262-265-266-277-282-285-288-289-292-296-297-299-301-303-304-306-307-308-315-316-318-319-320-323-324-325-326-327-328-332-336-337-338-339-342-343-346-347-348-349-351-358-360-363-375-376-401-402-403-404-405-410-411-412-413-423-430-434-435-438-442-445-452-453-459-460-464-465-469-470-471-473-474-482-491-492-501-502-504-505-506-517-519-520-522-523-530-531-533-539-541-548-549-550-551-560-561-563-581-582-585-588-589-590-592-593-597-607-608-619-622-627-630-631-635-636-637-639-642-647-648-650-651-652-656-657-659-660-666-667-669-670-679-680-690-691-692-693-698-700-704-705-706			
Mus musculus	2-3-8-9-10-11-13-14-15-16-17-18-20-36-37-39-45-46-56-57-61-71-76-77-82-83-91-103-107-117-121-122-123-124-126-127-129-130-131-139-144-149-154-162-163-169-170-173-181-183-184-187-198-213-214-215-216-218-225-226-228-232-233-236-239-242-246-247-255-261-264-265-276-281-287-288-289-290-295-296-298-299-303-304-305-306-307-314-315-317-318-319-324-325-327-331-335-	216	ACE inhibitor;antioxidative;dipeptidyl peptidase IV inhibitor;stimulating	26

	336-337-338-339-340-348-349-351-352-360-362-373-374-375-381-384-386-392-404-411-412-422-429-434-437-440-441-451-452-458-459-463-464-468-469-470-472-473-481-490-491-492-497-500-501-503-504-505-516-519-521-522-528-532-538-540-547-548-549-550-554-559-560-561-562-566-567-573-580-581-583-584-585-586-588-589-590-591-592-596-603-606-607-618-621-624-625-628-629-630-635-636-638-642-646-647-648-649-650-653-655-656-658-659-665-666-668-672-677-678-681-689-690-691-697-699-703-704-705			

Source: Prepared by the authors

ANEXOS

ANEXO 1- Comitê de ética

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

FLS. 33
PROC. 9202/16

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Proteoma da transição do colostro para o leite em cabras: interações proteicas para a maturação dos sistemas imunológico e digestivo do recém-nascido"**, Processo FOA nº 00422-2018, sob responsabilidade de Francisco Leydson Formiga Feitosa apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 14 de Agosto de 2018.

VALIDADE DESTE CERTIFICADO: 14 de Maio de 2020.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 14 de Junho de 2020.

CERTIFICATE

We certify that the study entitled **"Proteome from the transition from colostrum to milk in goats: protein interactions in maturation of the immune and digestive systems of the newborn"**, Protocol FOA nº 00422-2018, under the supervision of Francisco Leydson Formiga Feitosa presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on August 14, 2018.

VALIDITY OF THIS CERTIFICATE: May 14, 2020.
DATE OF SUBMISSION OF THE FINAL REPORT: June 14, 2020.

Prof. Ass. Dr. Leonardo Perez Faverani
Coordenador da CEUA
CEUA Coordinator

ANEXO 2 - Normas de Publicação da Revista - Small Ruminant Research

Small Ruminant Research publishes original, basic and applied research articles, technical notes, and review articles on research relating to **goats**, **sheep**, **deer**, the **New World camelids llama**, **alpaca**, **vicuna** and **guanaco**, and the **Old World camels**.

Topics covered include nutrition, physiology, anatomy, genetics, microbiology, ethology, product technology, socio-economics, management, sustainability and environment, veterinary medicine and husbandry engineering.

AUDIENCE

Research Scientists working on sheep, goats, deer and other small ruminants.

IMPACT FACTOR

2019: 1.273 © Clarivate Analytics Journal Citation Reports 2020

GUIDE FOR AUTHORS

Novelty: Novelty and relevance to an international readership will determine if a manuscript has merit to be published in Small Ruminant Research.

Types of article

1. Original Research Papers (Regular Papers)
2. Review Articles
3. Short Communication
4. Technical Notes
5. Short Technical Notes
6. Book Reviews

Original Research Papers should report the results of original research. The material should not have been previously published elsewhere, except in a preliminary form.

Review Articles should cover subjects falling within the scope of the journal which are of active current interest. Reviews will often be invited, but submitted reviews will also be considered for publication. All reviews will be subject to the same peer review process as applies for original papers.

A Short Communication is a concise but complete description of a limited investigation, which will not be included in a later paper. Submission of short communications is not encouraged. Short Communications may result from a request to condense a regular paper, during the peer review process. Results and Discussion are merged. Short Communications should not exceed 2,000 words. including figures, tables and references. The number of tables and figures should not exceed four.

A Technical Note is a report on a new method, technique or procedure falling within the scope of *Small Ruminant Research*. It may involve a new algorithm, computer program (e.g. for statistical analysis or for simulation), or testing method for example. The Technical Note should be used for information that cannot adequately incorporated into and Original Research Article, but that is of sufficient value to be brought to the attention of the readers of *Small Ruminant Research*. The note

should describe the nature of the new method, technique or procedure and clarify how it differs from those currently in use. It should not exceed 4,000 words.

Short Technical Notes of approximately 500 words can be submitted by geneticists to report the existence of genes and mutations found in small ruminants.

Book Reviews will be included in the journal on a range of relevant books which are not more than 2 years old. Book reviews will be solicited. Unsolicited reviews will not usually be accepted, but suggestions for appropriate books for review may be sent to the Editor-in-Chief.

What is publishable: Papers on polymorphism studies will be considered only if they contain significant new information and have direct relevance to those species described in the aims and scope of this journal. Submissions on studies involving single-nucleotide polymorphism (SNP) only, without linking them strongly and experimentally to production traits, are not encouraged. Manuscripts with quantitative RT-PCR without multiple normalizer gene products will be declined at preliminary review. Geneticists can submit Short Technical Notes of approximately 500 words, which will include the name of the gene, the location of the mutation (the sequence has to be deposited), the description of the population (breed, location, significant characters), possibly the allele frequency, even in small population, and some additional relevant information, with no need to demonstrate significant association with phenotypic traits or discussion. Accumulation of such information may lead to design comprehensive association studies in sheep and goats.
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ANEXO 3 - Normas de Publicação da Revista - Food Research International

We are pleased to announce that *Food Research International* has been accepted in MEDLINE as of March 7th, 2017.

Food Research International provides a forum for the rapid dissemination of significant novel and high impact research in food science, technology, engineering and nutrition. The journal only publishes novel, high quality and high impact review papers, original research papers and letters to the editors, in the various disciplines encompassing the science and technology of food. It is journal policy to publish special issues on topical and emergent subjects of food research or food researchrelated areas. Special issues of selected, peer-reviewed papers from scientific meetings, workshops, conferences on the science, technology and engineering of foods will be also published.

Food Research International is the successor to the Canadian Institute of Food Science and Technology Journal. Building on the quality and strengths of its predecessor, *Food Research International* has been developed to create a truly international forum for the communication of research in **food science**.

Topics covered by the journal include:
food chemistry food microbiology and **safety** microbiome food **toxicology materials** science of foods **food engineering** physical **properties** of foods **sensory science food quality** health and **nutrition food biophysics** analysis of foods **food nanotechnology emerging technologies**

Subjects that ***will not*** be considered for publication in *Food Research International*, and will be rejected as being outside of scope, include :
Studies testing different formulations and ingredients leading to the choice of the best formulation or ingredient to be used in the manufacture of a specified food; Optimization studies aiming to determine processing conditions and/or raw materials that increase the yield of a production process or improve nutritional and sensorial qualities; Studies describing the production of ingredients and only their characterization without a strong mechanistic emphasis; Studies describing the biological activity of foods lacking identification of the compounds responsible for the reported activity will not be published. This is also valid for any other chemical compounds such as phytochemicals and minor components of foods. Compounds of interest need to be characterized at least by mass spectrometrybased methods. Studies that do not clearly prove the relationship between the structure of the compounds and their activity; Fingerprinting studies lacking molecular insights and validation sets; Studies on antimicrobial compounds that do not consider a validation step in foods, lacking full data on chemical composition indicating the compounds responsible for the inhibitory activity and, when appropriate, the use of molecular biology approaches to support the findings; Development of analytical methods not comprising a validation step in situ that represent the range of conditions faced during their application will not be considered; Surveys of chemical, nutritional, physical and microbiological hazards will not be considered. Only papers presenting a significant data set, wide coverage, novel and supported by adequate chemical or microbiological techniques will be considered; Pharmacology and nutritional studies papers focusing in hosts rather than in foods. Pharmacology and nutritional studies that do not contain bioavailability or biofunctionality. Engineering studies lacking of mathematical verification or validation in situ, when appropriate; Fragmented studies, of low scientific quality, or poorly written. Studies with no food component.

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GUIDE FOR AUTHORS

INTRODUCTION

Food Research International is the successor to the Canadian Institute of Food Science and Technology Journal. Building on the quality and strengths of its predecessor, *Food Research International* has been developed to create a truly international forum for the communication of research in food science.

Food Research International provides a forum for the rapid dissemination of significant novel and high impact research in food science, technology, engineering and nutrition. The journal only publishes novel, high quality and high impact review papers, original research papers and letters to the editors, in the various disciplines encompassing the science and technology of food. It is journal policy to publish special issues on topical and emergent subjects of food research or food researchrelated areas. Special issues of selected, peer-reviewed papers from scientific meetings, workshops, conferences on the science, technology and engineering of foods will be also published.

Food Research International does not publish papers with a product development emphasis, statistical optimizations of processes or surveys. This is based on the editorial policy of the journal to publish more fundamental work with a strong quantitative emphasis and of a general nature.

Topics covered by the journal include:
Emerging Technologies Sensory Aspects of Foods Food Toxicology Food Chemistry and Analysis Food Omics Nutrition, health and food digestion Food Engineering and Materials Science of Foods Functional Foods Food Microbiology, Safety and Quality

Please also refer to the list of subjects not considered in *Food Research International* before you submit your paper. These topics can be found in the full aims and scope of the journal.

Types of paper

Research papers - original full-length research papers which have not been published previously, except in a preliminary form, and should not exceed 6,000 words. The word count refers to the text of the manuscript per se, i.e., references, figures and tables are not considered. Review articles - will be accepted in all areas of food science covered by the scope of the journal. Review articles focused on recent literature published (for example, over the previous 2-5 years) as well as comprehensive and definitive reviews will be considered.Review papers must contain critical assessment of literature and may also contains author's views on the subject. There are no word counts and reference numbers limit for review papers. Short communications - Food Research International does not publish short communication papers. Letters to the Editor - Letters are published from time to time on matters of topical interest. Book Reviews

Food Research International is concerned with safeguarding the rights and welfare of animals and human research subjects. Authors must provide a letter with the approval from the ethics committee from the respective University or research center where the study was performed.

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Contact details for submission

Submission for all types of manuscripts to *Food Research International* proceeds totally online. Via the Elsevier Editorial System (EES) website for this journal, https://www.editorialmanager.com/foodres/default.aspx, you will be guided step-by-step through the creation and uploading of the various files.

Questions regarding content of a proposed submission can be directed to: foodresearchinternational@gmail.com.

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Authors are encouraged to read the helpful notes on statistics applied in the planning of experiments and assessment of results in the field of food science and technology. The more important univariate and bivariate parametric and non-parametric methods, their advantages and disadvantages are presented in "Observations on the use of statistical methods in Food Science and Technology by Granato (http://www.sciencedirect.com/science/article/pii/S0963996913005723).

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Van der Geer, J., Hanraads, J. A. J., & Lupton, R. A. (2018). The art of writing a scientific article. *Heliyon*, *19*, e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.
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Cancer Research UK. Cancer statistics reports for the UK. (2003).
http://www.cancerresearchuk.org/ aboutcancer/statistics/cancerstatsreport/ Accessed 13 March 2003.
Reference to a dataset:
[dataset] Oguro, M., Imahiro, S., Saito, S., Nakashizuka, T. (2015). *Mortality data for Japanese oak wilt disease and surrounding forest compositions*. Mendeley Data, v1.
https://doi.org/10.17632/ xwj98nb39r.1.
Reference to a conference paper or poster presentation:
Engle, E.K., Cash, T.F., & Jarry, J.L. (2009, November). The Body Image Behaviours Inventory-3: Development and validation of the Body Image Compulsive Actions and Body Image Avoidance Scales. Poster session presentation at the meeting of the Association for Behavioural and Cognitive Therapies, New York, NY.

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