UNIVERSIDADE ESTADUAL PAULISTA - UNESP
FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS
CÂMPUS DE JABOTICABAL

EXPRESSÃO GÊNICA DIFERENCIAL E ANÁLISE PROTÉICA DO LEITE DE BÚFALAS SADIAS E COM MASTITE SUBCLÍNICA

Fernanda Tanamati
Zootecnista

2018

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Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutora em Zootecnia.

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DADOS CURRICULARES DO AUTOR

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“A vida é uma peça de teatro que não nos permite ensaios.
Por isso, cante, chore, dance, ria e viva intensamente,
antes que a cortina se feche e a peça termine sem aplausos”.

Charles Chaplin

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SUMÁRIO

Página

EXPRESSÃO GÊNICA DIFERENCIAL E ANÁLISE PROTÉICA DO LEITE DE BÚFALAS SADIAS E COM MASTITE SUBCLÍNICA....iii
RESUMO....iii
DIFFERENTIAL GENE EXPRESSION AND ANALYSIS OF MILK PROTEINS FROM DAIRY BUFFALO WITH AND WITHOUT SUBCLINICAL MASTITIS....iv
ABSTRACT....iv
CAPÍTULO 1 – Considerações gerais....1
1. Introdução....1
2. Revisão da literatura....2
2.1. Aspectos gerais da bubalinocultura....2
2.2. Mastite....3
2.3. Sistema imune inato....4
2.4. Expressão gênica no estudo da mastite....5
2.5. Abordagem proteômica no estudo da mastite....6
3. Referências....8
CAPÍTULO 2 - Differential expression of immune response genes associated with subclinical mastitis in dairy buffaloes....14
Abstract....14
1. Introduction....15
2. Material and Methods....16
2.1 Animals....16
2.2 Sampling....16
2.3 Gene expression....17
3. Statistical analysis....21
4. Results....22
5. Discussion....22
6. Conclusions....24
7. Acknowledgments....24
8. References....24
CAPÍTULO 3 - Characterization of the protein expression profiles of milk proteins in dairy buffaloes with and without subclinical mastitis....28
Abstract....28

1. Introduction 29
2. Material and methods 30
2.1 Animals and sampling 30
2.2 Proteome analysis 31
3. Data processing 33
4. Integrated gene annotations from the buffalo and bovine reference assemblies 33
5. Statistical analysis of mass spectrometry data 34
6. Gene Ontology 34
7. Results 35
8. Discussion 47
9. Conclusions 49
10. Acknowledgments 50
11. References 50

EXPRESSÃO GÊNICA DIFERENCIAL E ANÁLISE PROTÉICA DO LEITE DE BÚFALAS SADIAS E COM MASTITE SUBCLÍNICA

RESUMO – A mastite em búfalas leiteiras causa perdas econômicas devido à diminuição da produção de leite, além de ter um efeito negativo no bem-estar dos animais. Deste modo, foram realizados dois estudos: o objetivo do estudo I foi avaliar o nível de expressão dos genes da lactoferrina (*LTF)*, fator de necrose tumoral alfa (*TNF-α*), interleucina-1 beta (*IL-1β*), interleucina-8 (*IL-8*), e *toll-like receptors* 2 (*TLR-2*) e 4 (*TLR-4*) em búfalas Murrah com e sem mastite subclínica. Amostras de leite de 12 búfalas com mastite e 12 de búfalas sem mastite foram coletadas, para a extração de RNA, síntese de cDNA e validação dos perfis de expressão por qRT-PCR. O ΔΔCt foi estimado utilizando contrastes ortogonais da expressão dos genes alvo corrigidos para a expressão dos genes *housekeeping* entre os tratamentos. A expressão dos genes *TLR-2*, *TLR-4*, *TNF-α*, *IL-1β* e *IL-8* foi regulada positivamente em búfalos com mastite, enquanto que o gene *LTF* não apresentou expressão diferencial. O estudo desses genes da função imune que são ativos na glândula mamária é importante para o desenvolvimento de estratégias destinadas a preservar a saúde do úbere. O objetivo do estudo II foi avaliar as proteínas presentes no soro do leite de búfalas com e sem mastite subclínica, utilizando uma abordagem proteômica para identificar proteínas diferencialmente expressas e potencial biomarcador para a doença. Para isso, 16 amostras de leite de búfalas Murrah foram coletadas, sendo oito animais com e oito animais sem mastite subclínica. O método contagem espectral foi utilizado para quantificar a abundância relativa das proteínas individuais e os bancos de dados de proteínas anotadas para *Bubalus bubalis* e para *Bos taurus* foram utilizados na análise. Após integração das anotações genéticas das referências de búfalos e bovinos, foram identificadas 1.033 proteínas, das quais 156 proteínas foram diferencialmente reguladas entre animais sadios e infectados. Foram identificados 18 processos biológicos nos quais essas proteínas participam e a catelicidina-3 foi identificada como um potencial biomarcador da mastite subclínica. Os resultados são importantes para compreender melhor o comportamento da mastite na glândula mamária das búfalas e, portanto, podem auxiliar no diagnóstico precoce da doença.

Palavras-chave: *Bubalus bubalis*, glândula mamária, LC/MS-MS, proteômica, qRT-PCR

DIFFERENTIAL GENE EXPRESSION AND ANALYSIS OF MILK PROTEINS FROM DAIRY BUFFALO WITH AND WITHOUT SUBCLINICAL MASTITIS

ABSTRACT- Mastitis in dairy buffalo causes economic losses due to decreased milk production, along with having a negative effect on the animals' welfare. Thus, two studies were performed: the objective of study I was to evaluate the expression levels of lactoferrin (*LTF*), tumor necrosis factor alpha (*TNF-α*), interleukin-1 beta (*IL-1β*), interleukin- 8 (*IL-8*) and toll-like receptors 2 (*TLR-2*) and 4 (*TLR-4*) in Murrah buffaloes with and without subclinical mastitis. Milk samples were collected from 12 buffaloes with mastitis and 12 buffaloes without mastitis for RNA extraction, cDNA synthesis, and validation of expression profiles by qRT-PCR. The ΔΔCt was estimated using orthogonal contrasts of the target genes expression adjusted for the expression of the housekeeping genes between both treatments. The expression of the *TLR-2*, *TLR-4*, *TNF-α*, *IL-1β* and *IL-8* genes were upregulated in buffaloes with mastitis, while the *LTF* gene showed no differential expression. The study of these immune function genes that are active in the mammary gland is important for the development of strategies aimed at preserving the health of the udder. The objective of study II was to evaluate the proteins present in the milk whey from buffaloes with and without subclinical mastitis, using a proteomic approach to identify differentially expressed proteins and potential biomarkers for the disease. For this, 16 samples of Murrah buffalo milk were collected, comprised of eight animals with and eight animals without subclinical mastitis. The spectral counting method was used to quantify the relative abundance of the individual proteins, and the databases of proteins annotated for *Bubalus bubalis* and for *Bos taurus* were used in the analysis. After integrating the gene annotations from the buffalo and bovine references, a total of 1,033 proteins were identified, of which 156 proteins were differentially regulated between healthy and affected animals. 18 biological processes in which these proteins participate were identified, and cathelicidin-3 was identified as a potential biomarker of subclinical mastitis. These results are important to better understand the behavior of mastitis in the buffalo mammary gland, and in turn may aid in the early diagnosis.

Keywords: *Bubalus bubalis*, mammary gland, LC/MS-MS, proteomic, qRT-PCR

CAPÍTULO 1 – Considerações gerais

1. Introdução

A produção de leite tem proporcionado grande retorno econômico aos criadores de búfalas, que optam por esses animais não só pelo temperamento dócil, mas também, pela capacidade de adaptação e rusticidade. No entanto, a mastite é um grande entrave para a criação de búfalas leiteiras, e para outras criações, pois é uma doença que causa prejuízos econômicos para a indústria leiteira (JANZEN, 1970; HALASA et al., 2007).

Quando o animal é afetado pela doença, diversos mecanismos de defesa são acionados como tentativa de combate. O tecido da glândula mamária é protegido por duas formas de mecanismos de defesa imunológica, a imunidade inata ou capacidade de resposta não-específica e imunidade adquirida ou adaptativa (MEDZHITOV; JANEWAY, 1997). A primeira age nos estágios iniciais da infecção, é representada por barreiras anatômicas, solúveis e celulares, e responde imediatamente aos patógenos invasores (CARNEIRO et al., 2009). A segunda é desencadeada quando o patógeno não é completamente eliminado pelo sistema de defesa inato, reconhece determinantes antigênicos específicos de um patógeno e requer vários dias para se tornar capaz de exercer resposta protetora após a infecção (BANNERMAN, 2009). Uma melhor compreensão sobre os mecanismos de defesa da glândula mamária, através do estudo dos níveis de expressão de genes relacionados ao sistema imune inato quando o animal é afetado pela mastite, é importante para o desenvolvimento de estratégias para a manutenção da saúde do úbere, e assim, evitar alterações na qualidade ou produção do leite.

A disponibilidade de ferramentas de pesquisa em genômica, transcriptômica e proteômica possibilita o estudo da imunidade da glândula mamária e da patogênese da mastite com maiores detalhes (RAINARD; RIOLLET, 2006). A proteômica têm adquirido um importante papel, principalmente quanto à melhoria do conhecimento sobre a mastite (RONCADA et al., 2012). Estudos que visam a expressão diferencial de proteínas do leite com e sem mastite e a busca de biomarcadores, fornecem ferramentas para o diagnóstico precoce da doença, permitindo o bem-estar animal,

bem como, a qualidade e segurança do leite produzido (SMOLENSKI et al., 2011; BENDIXEN et al., 2011). Assim, objetivo do estudo 1, foi avaliar o nível de expressão dos genes *TNF-α*, *IL-1β*, *LTF*, *IL-8*, *TLR-2* e *TLR-4* no leite de búfalas saudáveis e diagnosticadas com mastite subclínica. O objetivo do estudo 2 foi caracterizar as proteínas presentes no soro do leite de búfalas, sadias ou diagnosticadas com mastite subclínica, além de identificar proteínas diferencialmente expressas e potencial biomarcador da mastite subclínica.

2. Revisão da literatura

2.1. Aspectos gerais da bubalinocultura

Os búfalos, originários do continente asiático, são considerados animais de tripla aptidão, uma vez que são capazes de produzir carne, leite e trabalho. De acordo com estimativas da FAO, em 2014, a população mundial de búfalos estava em torno de 194.463.729 cabeças, sendo a Índia o detentor da maior concentração desses animais. Nesse país, assim como em outros países da Ásia, os búfalos assumem grande importância no desenvolvimento social e econômico.

No Brasil, sua entrada se deu em meados de 1885, na Ilha do Marajó, Estado do Pará. A adaptação desses animais ao clima tropical foi um importante fator de contribuição para que os mesmos apresentassem desempenhos satisfatórios no país, e dos pouco mais de 200 animais introduzidos, resultaram em 495 mil cabeças em 1980 (BERNARDES, 2007).

De acordo com a FAO, em 2014, o número de búfalos no Brasil era de 1.319.478 cabeças, sendo as quatro raças bubalinas oficialmente reconhecidas pela Associação Brasileira de Criadores de Búfalos (ABCB) pertencentes a dois grupos, os búfalos de rio (Murrah, Mediterrâneo e Jafarabadi) e os búfalos de pântano (Carabao).

A raça Murrah se destaca na produção leiteira, esta que tem se tornado cada vez mais viável devido ao atrativo retorno econômico que proporciona. A produção de leite de búfala (*Bubalus bubalis*) constitui 13% da produção mundial de leite, sendo produzido principalmente na Ásia (FERREIRA et al., 2013). O leite de búfalas apresenta elevados teores de sólidos totais, com valores de teor de proteína de

3,91% e de gordura 6,87%, aproximadamente (TONHATI et al., 2000). Tais características garantem alto rendimento no processamento dos seus derivados. A região sudeste do Brasil apresenta o mercado de queijo de búfala mais competitivo do país. Nesta região, a bubalinocultura é voltada quase que totalmente à produção de leite, o qual é destinado a elaboração de derivados, principalmente o queijo tipo *mozzarella* (SENO et al., 2007), um produto de grande aceitação e procura pelos consumidores devido ao seu sabor e qualidades nutricionais.

2.2. Mastite

A mastite, também conhecida como mamite, é caracterizada como uma inflamação da glândula mamária, uma reação às alterações metabólicas e fisiológicas, traumas, ou invasão de agentes patogênicos (OVIEDO-BOYSO et al., 2007). A doença ocorre por invasão dos microrganismos pelo canal do teto, e uma vez que prossegue para a cisterna da glândula, passam a atuar diversos fatores solúveis e celulares (CARNEIRO et al., 2009).

Em bovídeos (vacas, ovelhas, cabras e búfalas leiteiras), pode se manifestar na forma clínica ou subclínica. Quando o animal apresenta a mastite clínica, sinais da doença podem ser diagnosticados por exames de rotina, ou seja, é possível observar o leite com grumos e/ou pus, e sintomas como edema, hipertermia, dor à palpação e endurecimento da glândula mamária (MESQUITA et al., 2017). Na mastite subclínica não há a manifestação de sintomas clínicos, assim como também não há alterações visíveis na aparência do leite ou úbere (ABEBE et al., 2016). Nesse caso, a detecção é realizada por testes específicos baseados no conteúdo celular ou alterações bioquímicas do leite. Dentre os diversos métodos de diagnóstico da mastite subclínica, os principais são: *Califórnia Mastitis Test* (CMT), cultura microbiológica e contagem de células somáticas (CCS).

Células somáticas são formadas por diferentes tipos celulares, incluindo neutrófilos, macrófagos, linfócitos, eosinófilos, e vários tipos de células epiteliais da glândula mamária (KEHRLI; SHUSTER, 1994). O aumento na contagem de células somáticas (CCS), principalmente de neutrófilos, ocorre no processo inflamatório, após a invasão e infecção do tecido da glândula mamária (OVIEDO-BOYSO et al.,

2007). De acordo com Cerón-Muñoz et al. (2002), a elevada CCS está associada com efeito negativo sobre a produção e qualidade do leite de búfalas.

Alterações na composição do leite provocadas pela mastite podem torná-lo menos adequado para o consumo e processamento e, assim causar grandes perdas econômicas para o setor (RAINARD; RIOLLET, 2006). Além da perda econômica e queda da qualidade do leite, a mastite também está associada a questões de bem-estar animal e saúde pública, já que, pode ocasionar resíduos no leite, devido ao uso impróprio de produtos antimicrobianos, e o surgimento de bactérias resistentes (DE VLIEGHER et al., 2012).

Sabe-se que em búfalas, características anatômicas do úbere e tetos, sistema de defesa atuante na glândula mamária e composição do leite conferem maior resistência contra a mastite, quando comparados com bovinos (SOLLECITO et al., 2011). Mesmo sabendo disso, para o controle da doença, as mesmas técnicas de criação e manejo indicadas para os bovinos são utilizadas (CARVALHO et al., 2007).

2.3. Sistema imune inato

Na resposta inflamatória à mastite, o sistema imune da glândula mamária é ativado para eliminar o patógeno. O sistema imune inato é um sistema de defesa imediato e inespecífico para controlar potenciais agentes infecciosos e não infecciosos (LINDE et al., 2008). Este sistema de resistência inclui neutrófilos, macrófagos, células epiteliais, mastócitos, eosinófilos e células NK (*natural killers*), que expressam uma variedade de receptores de reconhecimento padrão (OPPENHEIM et al., 2003).

Os patógenos apresentam estruturas de parede celular conhecidos como padrões moleculares associados a patógenos (PAMPs). Os PAMPs são reconhecidos por receptores específicos, denominados *Toll-like receptors* (TLRs), expressos nas células mamárias epiteliais e nos leucócitos presentes no leite (WELLNITZ; BRUCKMAIER, 2012). Em resposta ao reconhecimento, são liberadas ou ativadas diversas moléculas efetoras e mediadores de defesa (OPPENHEIM, et al., 2003), dentre as quais incluem os peptídeos antimicrobianos e mediadores químicos especializados (citocinas).

As citocinas pró-inflamatórias, fator de necrose tumoral alfa (*TNF-α*) e interleucina 1 beta (*IL-1β*), desencadeiam o processo inflamatório e promovem a ativação endotelial a fim de estimular a vasodilatação e o aumento da permeabilidade dos vasos sanguíneos, possibilitando o recrutamento das proteínas do soro e leucócitos para o local da infecção (MEDZHITOV, 2007). Além da *TNF-α* e *IL-1β*, diversas citocinas classificadas como interleucinas, têm sido encontradas nas glândulas mamárias, destacando-se a *IL-8, IL-2, IL-4, IL6, IL-10* e *IL-12*. A *IL-8* é uma quimiocina importante para o recrutamento de neutrófilos para a glândula mamária produzida por monócitos, linfócitos T, macrófagos, células endoteliais e epiteliais (LINDE et al., 2008).

A proteína lactoferrina apresenta propriedade antimicrobiana (YAMAUCHI et al., 2006), além de desempenhar atividades antitumoral e anti-inflamatória (ANAND et al., 2015). Sua propriedade antimicrobiana pode ocorrer por dois mecanismos: interação direta com o microrganismo infeccioso (GARCÍA-MONTOYA et al., 2012) ou sequestro dos íons de ferro livres evitando o desenvolvimento de bactérias que o utilizam como cofator metabólico (CARNEIRO et al., 2009). A lactoferrina pode ser encontrada em grânulos de neutrófilos, colostro, leite, fluido vaginal, sêmen, lágrimas, saliva, secreções nasais, bile e fluídos gastrointestinais (O'HALLORAN et al., 2010).

Desse modo, o estudo desses genes na presença de mastite ocasionada pela invasão por diferentes patógenos pode se tornar uma ferramenta para o desenvolvimento de metodologias para melhorar a saúde do úbere, sendo fundamental para isso, estabelecer técnica adequada para extração do material genético para obtenção de resultados confiáveis.

2.4. Expressão gênica no estudo da mastite

O RNA utilizado para o estudo de genes expressos na glândula mamária pode ser obtido a partir de biópsia mamária, amostras de tecido obtidas no momento do abate, ou a partir de células somáticas do leite. Segundo Boutinaud et al. (2002), enquanto que o isolamento do RNA utilizando biópsia mamária é um procedimento invasivo, o isolamento a partir de células somáticas do leite permite uma amostragem fácil e repetitiva sem prejudicar o tecido. A extração de RNA das células

do leite tem sido utilizada com sucesso em bovinos leiteiros (ALLUWAIMI et al., 2003; WELLNITZ et al., 2006), ao passo que ainda não foi determinado um protocolo específico para búfalas.

Além do tecido biológico utilizado para a extração, é essencial a obtenção de material genético de boa qualidade e em quantidade adequada para análises do perfil da expressão gênica através da técnica de transcrição reversa seguida pela PCR em tempo real (qRT-PCR) (GASPARINO et al., 2010). Com a tecnologia de qRT-PCR é possível a realização da amplificação e quantificação simultânea dos ácidos nucléicos, monitorando a reação a cada ciclo e determinando valores durante a fase exponencial da reação. *Cycle Threshold* (CT) é o ponto que detecta o ciclo no qual a reação atinge o limiar da fase exponencial, ou seja, é neste ponto que é possível a quantificação exata e reprodutível baseada na emissão da fluorescência (NOVAIS; PIRES-ALVES, 2004). Trata-se de uma técnica altamente sensível, que permite a quantificação de transcritos raros e pequenas alterações no padrão da expressão gênica (PFAFFL, 2001) e que apresenta ainda diversos benefícios sobre os outros métodos de quantificação da expressão gênica, dos quais se destacam: não requer manipulação pós-amplificação, 10.000 - 100.000 vezes mais sensíveis do que os ensaios de proteção de RNase e podendo ainda detectar uma única cópia de um transcrito específico (WONG e MEDRANO, 2005).

Dessa forma, a análise de expressão de genes usando qRT-PCR tem se tornado importante em pesquisas biológicas, pois permite a compreensão do padrão da expressão dos genes em diferentes situações, o que é importante para a caracterização de doenças, como é o caso da mastite em bovinos leiteiros (ALVA-MURILLO et al., 2012; GÜNTHER et al., 2012; GILBERT et al., 2013; WU et al., 2015).

2.5. Abordagem proteômica no estudo da mastite

As ciências ômicas integram diferentes áreas do conhecimento, como biologia molecular, bioquímica e bioinformática, com o objetivo de analisar de forma global os sistemas biológicos, utilizando técnicas experimentais, softwares e bancos de dados (ESPINDOLA et al., 2010).

Com a proteômica pode-se estudar o conjunto total de proteínas presentes em organelas sub celulares ou até ecossistemas, de forma descritiva e quantitativa, suas variações na população, mudanças em resposta ao desenvolvimento e ao meio ambiente, modificações pós-tradução e interações com outras proteínas e entidades moleculares (VALLEDOR; JORRIN, 2011).

De maneira geral, as metodologias empregadas em proteômica podem ser classificadas nos tipos *bottom-up* ou *top-down*. O *top-down* é um processo no qual as proteínas intactas são submetidas à análise por espectrometria de massas. O tipo *bottom-up* ou *shotgun*, inclui separação dos peptídeos obtidos após digestão de soluções proteicas complexas, seguida de análise por espectrometria de massas (MS, *mass spectrometry*). As abordagens *bottom-up* possuem muitas vantagens, como sensibilidade e reprodutibilidade, mesmo para proteomas complexos, como os de soro e lisados celulares (BARBOSA et al., 2012).

A primeira etapa a ser considerada na abordagem proteômica é a preparação das amostras, que deve ser realizada através do isolamento ou extração das proteínas a partir de amostras biológicas, seja tecido ou fluidos corporais. Em seguida, tem-se a separação das proteínas, que pode ser realizada por eletroforese bidimensional em gel de poliacrilamida com dodecil sulfato de sódio (2D-PAGE), cromatografia líquida de alta performance (HPLC) ou cromatografia líquida bidimensional (2D-LC) (COLANTONIO; CHAN, 2005).

A espectrometria de massas é utilizada quando a identificação da proteína é o objetivo final. Neste caso, são necessárias algumas etapas prévias que incluem digestão das proteínas, geralmente com tripsina, para digestão das proteínas em peptídeos, seguido de um processo de purificação (HPLC) dos peptídeos. A espectrometria de massas é o método atual de escolha para a identificação de proteínas, pois, este método oferece alta sensibilidade e capacidade para identificação de proteínas de alto rendimento (AEBERSOLD; MANN, 2003; CALANTONIO; CHAN, 2005).

A espectrometria de massa em tandem (MS/MS), é capaz de fornecer informação sobre a sequência de aminoácidos nos fragmentos peptídicos da proteína original, é a conexão de dois MS em série. O primeiro MS fornece informações de massa em cada peptídeo tríptico. O segundo MS desintegra cada

peptídeo e fornece uma medida dependente de tempo e massa que é usada para determinar o aminoácido que foi removido e, desse modo, fornece a sequência de aminoácidos para cada peptídeo (JAMESDANIEL et al., 2009).

Os espectros MS e MS/MS são adquiridos e armazenados para correspondência utilizando bases de dados de sequências de proteínas, e o resultado é a identificação dos peptídeos e, consequentemente, das proteínas (AEBERSOLD; MANN, 2003).

Dessa forma, a proteômica pode contribuir para o melhor conhecimento da biologia mamária e da lactação em ruminantes (FERREIRA et al., 2013). Trata-se de uma ferramenta importante para a caracterização das proteínas do leite, as quais possuem as mais variadas funções, como: portadoras de minerais ou vitaminas e outros compostos imobilizáveis e insolúveis quimicamente, estabilizadoras de grandes agregados ou micelas de lipídios e, finalmente, componentes de sistemas de defesa imune e inata (RONCADA et al., 2013). Assim, diversos estudos vêm sendo realizados com o objetivo de caracterizar possíveis alterações causadas pela mastite em animais leiteiros (YANG et al., 2009; ALONSO-FAUSTE et al., 2012; LI et al., 2014), ou até mesmo na busca de potenciais biomarcadores para esta doença (HOGARTH et al., 2004; MANSOR et al., 2013).

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CAPÍTULO 2 - Differential expression of immune response genes associated with subclinical mastitis in dairy buffaloes

Abstract - Buffalo milk production has become of significant importance on a world scale, however, there are few studies involving biotechnological tools specifically for buffalo. To characterize the innate immune system in the udder of dairy buffaloes, we evaluated the levels of expression of the lactoferrin (*LTF*), tumor necrosis factor alpha (*TNF-α*), interleukin-1 beta (*IL-1β*), interleukin-8 (*IL-8*) and toll-like receptors 2 (*TLR-2*) and 4 (*TLR-4*) genes in buffaloes with and without mastitis. Milk samples were collected for RNA extraction, cDNA synthesis, and quantification of expression profiles by qRT-PCR using specific primers. The ΔΔCt was estimated using orthogonal contrasts of the target genes expression adjusted for the expression of the housekeeping genes between both treatments. For *TLR-2*, *TLR-4*, *TNF-α*, *IL-1β* and *IL-8* genes, expression was upregulated in buffaloes with mastitis, while the *LTF* gene showed no differential expression. The study of these immune function genes that are active in the mammary gland is important to better understand the innate immunity that occurs in subclinical mastitis in dairy buffaloes and may aid the development of strategies to preserve the health of the udder.

Keywords: *Bubalus bubalis*, gene expression, innate immune system, milk, qRT-PCR

1. Introduction

Buffalo milk has a high total solids content, with an average protein content of 3.91±0.61% and fat content of 6.87±0.88% (Tonhati *et al*., 2000). These characteristics ensure a high yield in the processing of its derivatives. However, milk composition and milk production can be significantly affected by mastitis. Changes in milk composition caused by mastitis can make the milk less suitable for consumption and processing, with considerable economic losses to the dairy industry (Rainard and Riollet, 2006). The disease is characterized by inflammation of the mammary gland resulting from metabolic and physiological changes, trauma, or invasion of pathogens (Oviedo-Boyso *et al*., 2007). Several types of microorganisms have been associated with mastitis including mostly bacteria, but also fungi and yeast (Petzl *et al*., 2008).

Mastitis is either clinical or subclinical, the former of which can be easily diagnosed, whereas, with subclinical mastitis, there are no clinical symptoms or visible changes in milk or udder appearance (Abebe *et al*., 2016). Thus, control strategies are needed that depend on the early and accurate detection of subclinical mastitis, since early detection can reduce the negative effects of the disease and lead to higher cure rates (Deluyker *et al*., 2005; Shittu *et al*., 2012). The predominant defense in the early stage of mastitis is the innate immune system, also known as nonspecific immunity. The appropriate action of this defense mechanism promotes the rapid elimination of most pathogens (Sordillo and Streicher, 2002).

Pathogens have cell wall structures, known as pathogen-associated molecular patterns (PAMPs). PAMPs are recognized by specific receptors, called toll-like receptors (*TLRs*), including type 2 (*TLR2*) and type 4 (*TLR4*), which are expressed on mammary epithelial cells and leukocytes present in milk (Wellnitz and Bruckmaier, 2012). This recognition induces the release or activation of several effector molecules and defense mediators (Oppenheim *et al*., 2003), including antimicrobial peptides and specialized chemical mediators (cytokines).

Proinflammatory cytokines such as tumor necrosis factor alpha (*TNF-α*) and interleukin 1 beta (*IL-1β*) trigger an inflammatory response and mediate the recruitment of neutrophils from the bloodstream to the site of infection to eliminate the invading pathogens (Oviedo-Boyso *et al*., 2007). In addition to *TNF-α* and *IL-1β*, several cytokines classified as interleukins have been found in mammary glands,

including *IL-8*. Lactoferrin (*LTF*) is an antimicrobial peptide produced by epithelial cells, macrophages and neutrophils, which binds to free iron ions present in milk, thus preventing the growth of bacteria that use this ion as a metabolic cofactor (Carneiro *et al*., 2009).

The aim of this study was to evaluate the level of expression of the *TNF-α*, *IL-1β*, *LTF*, *IL-8*, *TLR-2* and *TLR-4* genes in the milk of healthy buffaloes and animals diagnosed with subclinical mastitis. The changes identified in gene expression levels will contribute to a better understanding of how the immune system responds in the presence of mastitis.

2. Material and Methods

2.1 Animals

Milk samples were collected from 24 Murrah buffaloes in the first third of lactation, in two days, from a farm located in Registro, São Paulo State, Brazil, supplied by the buffalo milk control program of the College of Agriculture and Veterinary Sciences of São Paulo State University (FCAV - UNESP). Twelve samples were collected from animals without mastitis (control group) and twelve from animals with mastitis (mastitis group).

2.2 Sampling

The milk samples were collected in the morning at the beginning of milking, following the routine procedure for buffalo cows. To determine which animals were positive or negative for subclinical mastitis we used the California Mastitis Test (CMT). The following criteria for the CMT were used: (-) there was no sign of infection, that is, the solution did not show precipitation, (+) positive for mastitis and presented with very viscous liquid and coagulated particles or presented a gelatinous and completely coagulated sample. After the CMT, milk aliquots were stored in flasks containing bronopol as a preservative and sent to the Milk Clinic at Luiz de Queiroz College of Agriculture of University of São Paulo (ESALQ-USP), Piracicaba, São Paulo State, Brazil, for somatic cell counting in a SomaCount 300® (Bentley Instruments, Inc.). The last sampling performed was for the RNA extraction, for which

approximately 150 mL of milk from each animal was divided into three sterile tubes, which were transported on disposable ice in isothermal containers.

The somatic cell score (SCS) was used for the classification of the groups for RNA extraction, in which ≤ 161 × 10^3 cells/mL was considered negative (animals without mastitis) and ≥ 860 × 10^3 cells/mL was considered positive (animals with mastitis). The SCS was compared between buffaloes with and without mastitis by the Tukey test, considering $P < 0.05$ as significant (Table 1). For the normalization of SCS, the data were log transformed [log2(SCS/100,000)] + 3 as described by Dabdoub and Shook (1984).

Table 1 *Somatic cell score (SCS) in the milk of buffalo with and without mastitis*

	Mastitis group				Control group				P-value
	Min.	Max.	Mean	SD	Min.	Max.	Mean	SD	
SCS (x 10^3)	860.0	4,159.0	2,240.0[a]	1,360.5	15.0	161.0	87.3[b]	46.7	$6.3e^{-10}$

Min. = minimum, Max. = maximum, SD = standard deviation
[a,b] Values within a row with different superscripts differ significantly at P<0.05.

2.3 Gene expression

Total RNA was extracted with the Trizol® reagent (Invitrogen, USA). All materials were sterile and free of RNase or were previously treated with RNaseZAP® (Invitrogen, EUA) to prevent RNA degradation. The somatic cells were isolated from milk by centrifugation at 12,000 × *g* for 10 min at 4ºC, followed by the addition of 1 mL of Trizol®, homogenization and freezing at -80°C for subsequent RNA extraction according to manufacturer recommendations. One week later, after thawing in the refrigerator for 4 hours, the material was briefly homogenized and centrifuged at 12,000 × *g* for 12 min at 4ºC. The supernatant was transferred to a new tube and incubated for 5 min at room temperature. Next, 200 µL of chloroform was added and the samples were shaken before incubation for an additional 3 min at room temperature, followed by centrifugation at 12,000 × *g* for 15 min at 4ºC. The aqueous phase was collected and transferred to a new tube and the same volume of isopropanol was added. The samples were homogenized by inversion, incubated for

10 min at room temperature, and centrifuged at 12,000 × *g* for 10 min at 4ºC. The supernatant was discarded, and the precipitate was washed with 1 mL of 75% ethanol. The samples were again centrifuged at 7,500 × *g* for 5 min and the supernatant was discarded. The pellet was dried for 30 min at room temperature, resuspended in RNase-free ultrapure water, and incubated for 15 min in a water bath at 55-60ºC for 15 min. After this last step, the samples were stored in a freezer at -80ºC.

The concentration and purity of RNA were measured in a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) at 260 and 280 nm, and in a Qubit® 2.0 Fluorometer (Invitrogen, USA). The absence of contamination of the samples with genomic DNA was confirmed in a Qubit® 2.0 Fluorometer (Invitrogen, USA) using the Qubit dsDNA Assay Kit (Invitrogen, USA). The results were considered satisfactory when the amount of genomic DNA measured with the Qubit fluorometer was less than 10% of the total nucleotides in each sample. When the percentage exceeded the acceptable limit, the samples were treated with the TURBO DNA-free™ Kit (Life Technologies, USA).

The cDNA was synthesized using the SuperScript IV First-Strand cDNA Synthesis Reaction Kit (Invitrogen, USA) according to manufacturer recommendations. SuperScript IV can be used for cDNA synthesis of any type of RNA and has a high efficiency with degraded and limited RNA. For the assay, 1 µL of oligo(dT)20 primer (50 µM), 1 µL of dNTPs (10 mM) and 2 µL of total RNA were mixed and RNase-free ultrapure water was added to reach a volume of 13 µL. The reaction was incubated for 5 min at 65ºC and kept on ice for at least 1 min. Next, 4 µL of 5X SSIV buffer, 1 µL of ribonuclease inhibitor, 1 µL of DTT (10 mM), and 1 µL of (200 U) SuperScript IV were added. The mixture was incubated at 55ºC for 10 min and at 80ºC for an additional 10 min. The synthesized cDNA was stored at -20ºC until use.

The primers used in the amplification reactions for the study of gene expression were constructed using buffalo gene sequences obtained from GenBank (htpp://www.ncbi.nlm.nih.gov/genbank/) (Table 2). The primers for the *TLR-2*, *TLR-4*, *LTF*, *TNF-α*, *IL-8* genes and for the housekeeping gene (*EEF1A1*) were obtained from studies on cattle and were redesigned using the homologous sequences in

buffaloes. The primers for the *IL-1β*, *GAPDH* and *RPLP0* genes were obtained from the literature (Table 2). Comparison of the primers sequences with the sequence of the corresponding gene in buffaloes showed 100% identity and it was therefore not necessary to redesign these primers. The mRNA sequence of the *HPRT1* gene expressed in buffaloes was used to design the primer to avoid annealing to intron regions. The Primer Express 3.0 software (Applied Biosystems, USA) was used to design the primers, taking into consideration the size of the primer, size of the amplified product, percentage of cytosine and guanine, and melting temperature (T_M), as well as the absence of dimers and hairpin formation. The selected primer sequences were aligned using the BLAST algorithm (Basic Local Alignment Search Tool) of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST) to identify regions in the selected sequences that would show a high degree of identity with other regions of the genome or within a gene family.

Table 2 *Sequence of forward (F) and reverse (R) primers for qRT-PCR*

Genes	Primer sequence (5'→3')	Amplicon size (bp)	References
TLR-2	F: CAGTTTAACCCAGTGCCTTC R: CTCCAAAGTCTTCAGCTGCT	241	Redesigned primer
TLR-4	F: TGCCTCCACTACAGGGACTTT R: TGGGACACCACGACAATAAC	101	Redesigned primer
LTF	F: GGCCTTTGTCTTGGAATGTATC R: ATTTAGCCACAGCTCCCTGGAG	338	Redesigned primer
TNF-α	F: TCTTCTCAAGCCTCAAGTAACAAGC R: CCATGAGGGCATTGGCATAT	103	Redesigned primer
IL-1β	F: AGTGCCTACGCACATGTCTTC R: TGCGTCACACAGAAACTCGTC	114	Griesbeck-Zilch et al., 2008
IL-8	F: ACACATTCCACACCTTTCCA R: GGTTTAAGCAGACCTCTTTT	124	Redesigned primer
GAPDH	F: CCGTTCGACAGATAGCCGTAA R: CGACCTTCACCATCTTGTCTCA	69	Fonseca et al., 2015b
EEF1A1	F: CATCCCAGGCTGACTGTGC R: TATAAGCCAGAAGGGCATGC	108	Redesigned primer
RPLP0	F: CAACCCTGAAGTGCTTGACAT R: AGGCAGATGGATCAGCCA	227	Mount et al., 2009, Fonseca et al., 2015a
HPRT1	F: GAGAGTCCGAGTTGAGTTTGG R: AGAACGAGAACATTGGATCATG	76	GenBank XM_006074802.1

The concentrations of the primers were determined by titration: 300 nM forward and reverse primers for *EEF1A1* and *RPLP0*, and 100 nM forward and reverse primers for *GAPDH*, *HPRT1*, *IL-1β LTF*, *TLR-2*, *TLR-4*, *TNF-α* and *IL-8*. For all genes the qRT-PCR efficiency was calculated by a standard curve from serial cDNA dilution (2:1). Efficiency between 90 and 110% was considered for PCR primers (Livak and Schmittgen, 2001).

A total of four candidate housekeeping genes (*GAPDH*, *EEF1A1*, *RPLP0*, and *HPRT1*) were analyzed using the GeNorm algorithm (Vandesompele *et al*., 2002) and the PROC MIXED procedure of the Statistical Analysis System software (SAS Institute, Inc., Cary, NC), considering treatment as a fixed effect and biological repetition as a random effect. The *EEF1A1* and *RPLP0* housekeeping genes were

identified as the most stable, and the geometric mean of these genes (Vandesompele *et al*., 2002) was used for each sample in this study.

Real-time PCR was performed with the 7500 Real-Time PCR system (Applied Biosystems, USA) using the SYBR Green Master Mix (Applied Biosystems, USA), at a final volume of 12.5 µL. All the reactions were performed in triplicate and the amplification conditions were: 2 min at 50ºC, 10 min at 95ºC, and 40 cycles at 95ºC for 15 sec and at 60ºC for 1 min. The melting curve of the reaction products was obtained.

3. Statistical analysis

The cycle threshold (Ct) values were obtained with the Expression Suite software v1.0 (Applied Biosystems, USA). The analysis was performed with the PROC MIXED procedure of the Statistical Analysis System software (SAS Institute, Inc., Cary, NC), in which the following mixed linear model was adjusted:

$$Y_{gikr} = TG_{ig} + G_k + P_k + D_{ik} + e_{gikr}$$

where Y_{gikr} is the Ct obtained from the thermocycler software for the g^{th} gene (target and housekeeping genes) located in the r^{th} well of the microplate (referring to the technical replicate), corresponding to the k^{th} animal and the i^{th} treatment (with or without mastitis); TG_{ig} is the fixed effect of the *i* treatment in the expression of gene *g*; G_k is the random effect of the genes for animal *k*; P_k is the random effect of the microplate for animal *k*; D_{ik} is a random sample-specific effect that captures differences between samples common to the genes, particularly those affecting RNA concentration such as extraction and amplification efficiency, and e_{gikr} is the residual effect.

Using the model described above, an approximation of $\Delta\Delta$Ct was estimated as proposed by Steibel *et al*. (2009). This approach also permits the inference of the level of significance of the difference in the transcription of these genes using orthogonal contrasts between treatments and the housekeeping and target genes. Approximated $\Delta\Delta$Ct values with a P- value less than 5% ($P < 0.05$) were considered significant.

4. Results

The mRNA expression of *TLR-2*, *TLR-4*, *TNF-α*, *IL-1β*, *IL-8* and *LTF* genes were studied in the milk of buffaloes with and without subclinical mastitis (Table 3). The *TLR-2* gene showed expression 13.34 times higher in animals with mastitis compared to animals without mastitis. For the *TLR-4* gene, mRNA expression was also higher (7.49 times) in buffaloes with mastitis.

Table 3 *Approximation of ΔΔCt of the TLR-2 (toll-like receptor 2), TLR-4 (toll-like receptor 4), LTF (lactoferrin), TNF-α (tumor necrosis factor alpha), IL-1β (interleukin-1 beta) and IL-8 (interleukin-8) genes in the milk of buffalo with and without mastitis*

Genes	Estimate	Standard error	P-value
TLR-2	-3.7383	0.5773	<0.0001
TLR-4	-2.9043	0.5997	<0.0001
LTF	-0.4973	0.6211	0.4331
TNF-α	-2.8372	0.8850	0.0053
IL-1β	-5.9656	1.2107	0.0001
IL-8	-4.7118	0.8754	<0.0001

Expression of the *TNF-α, IL-1β* and *IL-8* genes differed significantly between the groups with and without mastitis. The relative expression of the *TNF-α*, *IL-1β* and *IL-8* genes was, respectively, 7.15, 62.49 and 26.20 times higher in animals with mastitis. No significant difference between groups was observed for the *LTF* gene.

5. Discussion

The results show that mRNA expressions of the *TLR-2* and *TLR-4* were higher in buffalo cows with mastitis, resulting in the association of these genes with immune system alterations caused by mastitis in dairy buffaloes, as were also observed in cattle (Goldammer *et al*., 2004, Griesbeck-Zilch *et al*., 2008, Fonseca *et al*., 2015a). Toll-like receptors are the first-line host response to the presence of pathogens and are responsible for triggering the innate immune response in animals (Pasare and Medzhitov, 2004).

After activation of toll-like receptors, different molecules and defense mediators are released or activated (Oppenheim *et al*., 2003), such as *TNF-α*, *IL-1β*,

IL-8 and *LTF,* all of which were evaluated in this study. The *TNF-α* and *IL-1β* genes encode proinflammatory cytokines that trigger the process of inflammation. Both cytokines mediate endothelial activation to stimulate vasodilation and an increase in blood vessel permeability, promoting the recruitment of serum proteins and leukocytes to the site of infection (Medzhitov, 2007). In this context, the increase in mRNA expression of both genes in animals with mastitis is expected since it is associated with protection against and elimination of the invading pathogen in the mammary gland. For this and other reasons, *TNF-α* and *IL-1β* genes are considered key components of the innate immune system (Lahouassa *et al*., 2007). As observed in cattle by Riollet *et al*. (2001), Pfaffl *et al*. (2003), Sarikaya *et al*. (2006), Griesbeck-Zilch *et al*. (2008) and Fonseca *et al*. (2015a), higher mRNA expression of these cytokines was also observed in animals infected with mastitis.

The *IL-8* gene encodes a chemokine (chemotactic cytokine) that attracts neutrophils to the site of infection. This cytokine is produced by a variety of cells, such as monocytes, T lymphocytes, macrophages and epithelial and endothelial cells (Oviedo-Boyso *et al*., 2007), and its expression can be induced by proinflammatory stimuli such as *TNF-α* and *IL-1β* (Mukaida, 2003). We may therefore suggest that the increased *IL-8* mRNA expression in animals with mastitis is related to the increased mRNA expression of *TNF-α* and *IL-1β* genes, increasing the number of neutrophils and, consequently, of somatic cells. Increased mRNA expression of the *IL-8* gene has also been reported for cattle (Zhu *et al*., 2012; Fu *et al*., 2013), suggesting that the mechanism of combating the pathogen seems to be similar in both species.

The *LTF* gene, the only gene for which no significant difference was observed between groups, encodes the glycoprotein lactoferrin that is part of the complement system of the innate immunity (Rainard and Riollet, 2006). This protein has been shown to exert antimicrobial activity (Yamauchi *et al*., 2006), as well as antitumor and anti-inflammatory properties (Anand *et al*., 2015). Thus, the expected result would be increased mRNA expression of this gene in the group with mastitis, which was not observed. According to Berlutti *et al*. (2006), lactoferrin downregulates proinflammatory cytokines, i.e., it prevents the release of inflammatory mediators and inhibits the production of cytokines (Kushibiki *et al*., 2011). However, the opposite effect seems to have occurred in the present study. It is possible that the differential

expression of the cytokines may have affected *LTF* mRNA expression, however, further studies are needed to confirm this result. The non-differential expression of *LTF* mRNA may also be the result of the changes caused by mastitis in mammary tissue, which could have affected epithelial cells that, together with leukocytes, are responsible for its production (Sordillo and Streicher, 2002).

The rapid and effective action of the studied genes, that participate in the nonspecific defense mechanism, is the first line of defense against pathogenic organisms and could eliminate them rapidly before the adaptive immunity be activated (Carneiro et al., 2009). In contrast to the innate immunity that is activated within a few hours, the adaptive immune system requires several days to act against infection (Bannerman, 2009). The rapid elimination of pathogens may prevent changes in the quality or produced milk.

6. Conclusions

Subclinical mastitis promotes changes in the expression of the *TLR-2*, *TLR-4*, *TNF-α*, *IL-1β* and *IL-8* genes in dairy buffaloes. This study contributes to a better understanding of the innate immunity that occurs in subclinical mastitis in dairy buffaloes.

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CAPÍTULO 3 - Characterization of the protein expression profiles of milk proteins in dairy buffaloes with and without subclinical mastitis

Abstract - The aim of this study was to characterize the proteins present in milk whey from buffaloes with and without subclinical mastitis, using a proteomic approach to identify differentially expressed proteins and potential biomarkers for the disease. A total of 16 milk samples were collected from Murrah buffaloes, which comprised eight animals with and eight animals without subclinical mastitis. The annotated protein databases for *Bubalus bubalis* and for *Bos taurus* were used in the analysis and spectral counting was used to quantify the relative abundance of individual proteins. After integrating gene annotations from the buffalo and bovine references, a total of 1,033 proteins were identified, of which 156 were differentially expressed. Eighteen biological processes were annotated with Gene Ontology. The genes responsible for encoding the differentially expressed proteins were also used for network and key player analyses. Cathelicidin-3 was identified as one of the three central genes in a network analysis, and as a potential biomarker of subclinical mastitis. These results are important to further understand the behavior of mastitis in the buffalo mammary gland and may also aid in early diagnosis.

Keywords: *Bubalus bubalis*, cathelicidin-3, LC-MS/MS, mammary gland, proteomic

1. Introduction

Milk is an important source of nutrients and a key ingredient in the production of several dairy food products. The high levels of protein and fat in the milk of buffalo, approximately 3.91% and 6.87%, respectively [1], assure high yields from the processing of the milk and its derivatives. However, diseases such as mastitis change the milk composition, making it less suitable for processing and human consumption and significantly reducing the value of the affected milk.

Mastitis is characterized as an inflammation of the mammary gland, a reaction to metabolic and physiological changes, trauma, or invasion of pathogenic agents [2] and is mainly caused by bacterial infection but can also be caused by microorganisms such as fungi and yeasts [3].

In healthy animals, somatic cells found in milk include neutrophils, macrophages, lymphocytes, eosinophils, and several types of epithelial cells from the mammary gland [4]. An increase in somatic cell count (SCC), mainly neutrophils, occurs in the inflammatory process after invasion and infection of the mammary gland tissue by a pathogenic agent [2]. In this context, strategies for the preservation and improvement of the health of the udder can be obtained through studies using various biotechnological tools.

The "omics" technologies, such as genomics, transcriptomics, proteomics and metabolomics, have undergone major development in recent years and are increasingly being applied in livestock research. The term proteomics was introduced by Marc Wilkins in 1995 [5] and can be defined as the large-scale characterization of the entire protein complement of a cell line or tissue [6,7,8]. Through this scientific approach, it is possible to identify the total number of genes expressed in a given tissue, verifying the level of protein expression, as well as predicting their function and tissue expression locations [9].

In dairy animals, proteomics has widely been used to analyze the milk fat globule membrane (MFGM) proteins [10], for benchmarking proteins among different mammalian species [11] or for the investigation of the effects of high somatic cell count on the milk proteins [12]. Furthermore, other studies have used proteomics for the identification of protein biomarkers in bovine milk with mastitis [13,14], and

comparisons between the proteins in milk produced by bovine cows with and without mastitis have been performed [13,15].

Studies that have searched for protein biomarkers via the differential expression of milk proteins in animals with and without mastitis assist in the characterization of economic losses due to mastitis and provide tools for the early diagnosis of infection, enabling improved animal welfare as well as ensuring the quality and safety of produced milk.

The aims of this study were to verify protein profile changes present in the milk whey from buffaloes with and without subclinical mastitis, to determine which of the proteins are differentially expressed and to identify any potential biomarkers of subclinical mastitis to aid in the early detection of this disease.

2. Material and methods

2.1 Animals and sampling

A total of 16 milk samples were collected from Murrah buffaloes from a farm located in Registro, São Paulo State, Brazil, supplied by the Buffalo Milk Control program of the São Paulo State University (FCAV - UNESP). Eight samples were collected from healthy animals (control group, without mastitis) and eight from animals with mastitis (mastitis group).

The method used to determine which animals were healthy or affected by subclinical mastitis was the California Mastitis Test (CMT). The criteria for the CMT was as follows: (-) negative for mastitis, that is, the solution did not show precipitation or presented slight precipitation that disappeared after agitation and, (+) positive for mastitis and presented a very viscous liquid or gelatinous sample with partially or completely coagulated particles.

Milk samples were collected for somatic cell count (SCC) and proteome analysis. For SCC, milk aliquots were stored in flasks containing bronopol as a preservative and sent to the Milk Clinic at Luiz de Queiroz College of Agriculture of University of São Paulo (ESALQ-USP), Piracicaba, São Paulo State, Brazil. For proteomic analysis, 20 mL of milk from each animal was collected in a sterile tube and transported on dry ice in isothermal containers, then stored in a freezer at -80°C.

The SCC method was used to group the animals considering ≤ 101 × 10^3 cells/mL for buffaloes without mastitis, and ≥ 2,310 × 10^3 cells/mL for buffaloes with mastitis.

2.2 Proteome analysis

2.2.1 *Sample preparation:* Laboratory analyses were performed at the Veritas Life Sciences biotechnology company at USP Technology Park of Ribeirao Preto, São Paulo State, Brazil. First, the samples were thawed in the refrigerator for 6 hours. Then, 1 mL of each sample was used and 10 uL of Protease Inhibitor Cocktail (Sigma-Aldrich, USA) was added. Samples were centrifuged at 3,000 × g for 15 minutes at 4°C for the removal of milk fat. The resulting skimmed milk was subjected to further centrifugation at 13,000 × g for 4 hours to obtain the whey proteins in the supernatant. About 500 µL of each sample was subjected to casein precipitation, using 50 µL of 0.5 M acetic acid to obtain pH = 4.6 (Figure 1). Centrifugation was performed at 3,000 × g for 5 minutes at 4°C and the supernatant was separated for proteomic analysis.

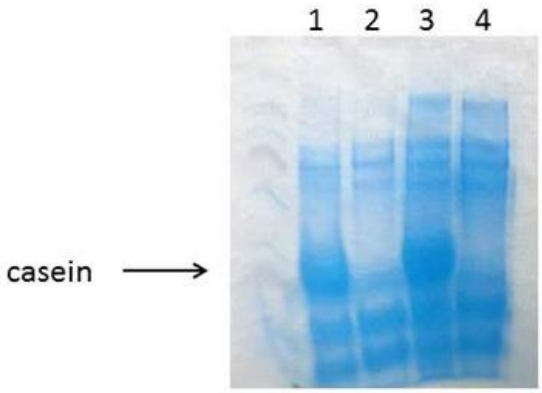


Figure 1. Casein precipitation of buffalo milk whey protein. Samples 1 and 3 before casein precipitation, samples 2 and 4 after casein precipitation.

For the protein quantification, the Bradford method was performed using the Protein Assay Dye Reagent Concentrate (Bio-Rad, USA). The standard curve was calculated from different concentrations of bovine serum albumin, prepared from a commercially available Protein Standard (Sigma-Aldrich, USA). The samples were distributed in microplates in triplicates of 1 µL each. Absorbance at 595 nm was read

in a SpectraMax Plus 384 spectrophotometer (Molecular Devices, USA). The total mass of protein present in each sample was estimated from the quantification.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to evaluate the protein profile. About 20 µg of each sample was resuspended in electrophoresis XT Sample Buffer 1X (Bio-Rad, USA) containing 20 µg of dithiothreitol (DTT) (GE Healthcare Life Sciences, USA) to reduce the disulfide bonds of the proteins. After incubation at 100°C for 5 minutes, the samples were alkylated with 100 µg of iodoacetamide (I.A.) (BioUltra/Sigma-Aldrich, USA) for 20 minutes at room temperature in the dark. Novex™ 12% Tris-Glycine gels (Invitrogen, USA) were used for electrophoresis which was performed with 40 V/gel for 30 minutes followed by 70 V for 2 hours in the Mini-PROTEAN II Electrophoresis System (Bio-Rad, USA). 5 µL of a PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific, USA) was used to determine the molecular weights of proteins. After separation by SDS-PAGE, gels were stained with Coomassie GelCode™ Blue Stain Reagent (Thermo Scientific, USA) for visualization of the proteins, according to the manufacturer's recommendations.

2.2.2 *LC–MS/MS analysis:* About 50 µg of each sample was prepared for mass spectrometry which consisted of three main steps: i) reduction and alkylation of proteins, ii) enzymatic digestion of the proteins with trypsin and iii) "clean up/desalting" of the samples. Disulfide bonds of the proteins present within each sample were reduced by the addition of DTT in the proportion of 1 mg DTT/mg protein and incubation for 2 hours at room temperature. This was followed by the addition the alkylating agent I.A. in the proportion of 3 mg I.A./mg protein and incubation for 1 hour at room temperature in the dark. The sample volume was diluted five times in 0.1 M of solution ammonium bicarbonate pH ≥8.0 to achieve a final volume of 500 µL. The samples were incubated with 1 µg of Sequencing Grade Modified Trypsin (Promega, USA) at 37°C overnight. Prior to sample analysis in the mass spectrometer, the samples were "cleaned-up"/desalted" using OASIS HLB Cartridge 1cc column (Waters, USA), according to the manufacturer's description. Briefly, the column was equilibrated with 5% acetonitrile solution containing 0.1% formic acid and the elution of the material of interest was performed with 80% acetonitrile. Samples were dried in a speed-vac and analyzed using a LTQ Orbitrap

ELITE mass spectrometer (Thermo-Finnigan, USA) coupled to a nanoflow chromatography system (LC-MS/MS). About 1 µg of each sample was used for analysis.

The liquid chromatography separation was performed in a 25 cm column (Picofrit 75µm ID, New Objective), packed in house with Magic C18, using a 60 minute linear gradient from 5 to 40% of acetonitrile in 0.1% formic acid at 250 nL/min. The spectra were acquired in a data-dependent mode in m/z range of 400 to 1800, and the 5 most abundant +2 or +3 ions of each MS spectrum were selected for MS/MS analysis. The mass spectrometer parameters were: capillary voltage of 2.2KV, capillary temperature of 200ºC, resolution of 100,000 and FT target value of 2,000,000.

3. Data processing

Data obtained were automatically processed by the "Computational Proteomics Analysis System - CPAS" [16], using the X!Tandem search algorithm. The minimum criteria for peptide matching was a PeptideProphet score greater than 0.9. The identified peptides were grouped into proteins, using the ProteinProphet algorithm which was used to generate a list of identifications with an error rate less than 2.0%. The annotated protein databases for *Bubalus bubalis* available on NCBI database (https://www.ncbi.nlm.nih.gov/protein) and annotated protein database for *Bos taurus* available on UNIPROT database (http://www.uniprot.org/) were used for this analysis.

The comparative and quantitative analysis was performed using the number of peptide-spectrum matches observed per peptide, which provides an estimation of abundance, also known as "spectral counting".

4. Integrated gene annotations from the buffalo and bovine reference assemblies

Gene annotations from the buffalo and bovine reference assemblies were considered. The water buffalo has two sequenced genomes [17,18] available at NCBI database and a reference set of transcripts using RNA-seq from 30 different tissues

[19], however, the buffalo genome is not fully annotated. To compensate for this, the bovine annotation was also considered since it is better characterized than buffalo.

The annotated proteins identified for buffalo and bovine were organized according to their respective gene symbol, and the genes symbols were grouped according to their spectral counts. If gene isoforms were on the same annotation and had the same number of counts, only one was considered. When the same gene symbol was identified for bovine and buffalo, the buffalo annotation was considered, whether the counts were the same or not. For buffalo and bovine gene isoforms that presented different counts, graphs were plotted and the trendline and R-square ($R \geq 0.95$) values were considered.

5. Statistical analysis of mass spectrometry data

Statistical analysis of differentially expressed proteins (DEP) was performed using the msmsTests package on R/Bioconductor [20], in which the negative binomial of the edgeR package is available. The multiple testing correction via false discovery rate (FDR) estimation was used considering $FDR<0.05$.

6. Gene Ontology

Gene Ontology Consortium (http://geneontology.org/) powered by Protein Analysis through Evolutionary Relationships (PANTHER) Classification System v12.0 was used to interpret the biological functions of the differentially expressed proteins (DEP). PANTHER Overrepresentation Test (release 20170413) was used for the statistical analysis considering only $P<0.05$, which was calculated by the Binomial statistic and Bonferroni correction.

The ‘Mutual Information NETworks (MINET)’ package [21] of R/Bioconductor was used for network analysis. The ‘keyplayer’ package [22] was also used to identify the most central group of genes/ proteins in a network.

A normal distribution model was used to select the potential biomarker. Due to the distinction of the counts between the control and mastitis groups and considering the standard error of the mean and standard deviation of these groups, the potential biomarker of subclinical mastitis was identified [23].

7. Results

A total of 1,993 proteins were identified, of which 1,044 were identified through the buffalo reference assembly and 949 using the bovine reference assembly (Figure 2).

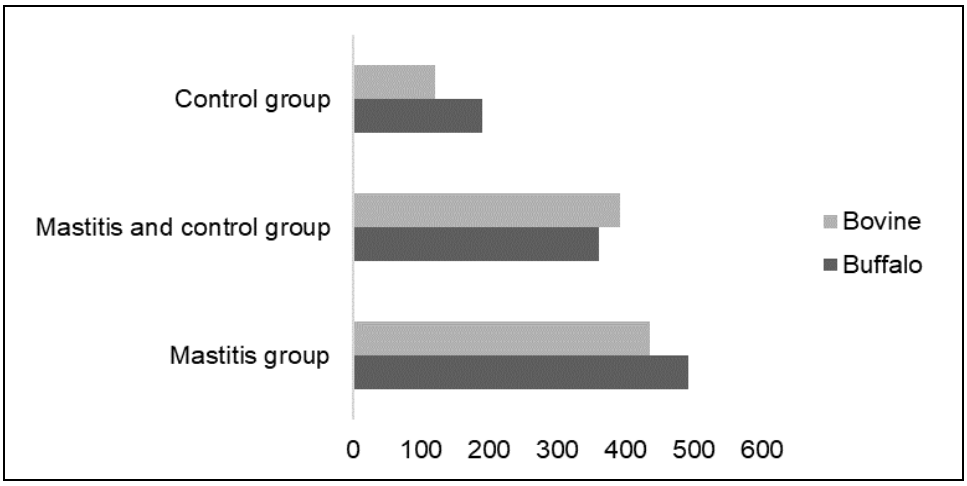


Figure 2: Proteins identified in milk whey samples of buffaloes with and without subclinical mastitis using buffalo and bovine reference assembly.

After integration of buffalo and bovine reference assemblies using the gene symbol, the total number of proteins identified was 1,033 (Figure 3), of which 252 were identified from buffalo/bovine reference genome, 304 unique to the bovine reference genome and 477 unique to the buffalo reference genome. The similarity between the number of proteins identified exclusively for the mastitis group, mastitis and control group, and exclusively for the control group was observed before and after integration (Figure 2 and Figure 3B).

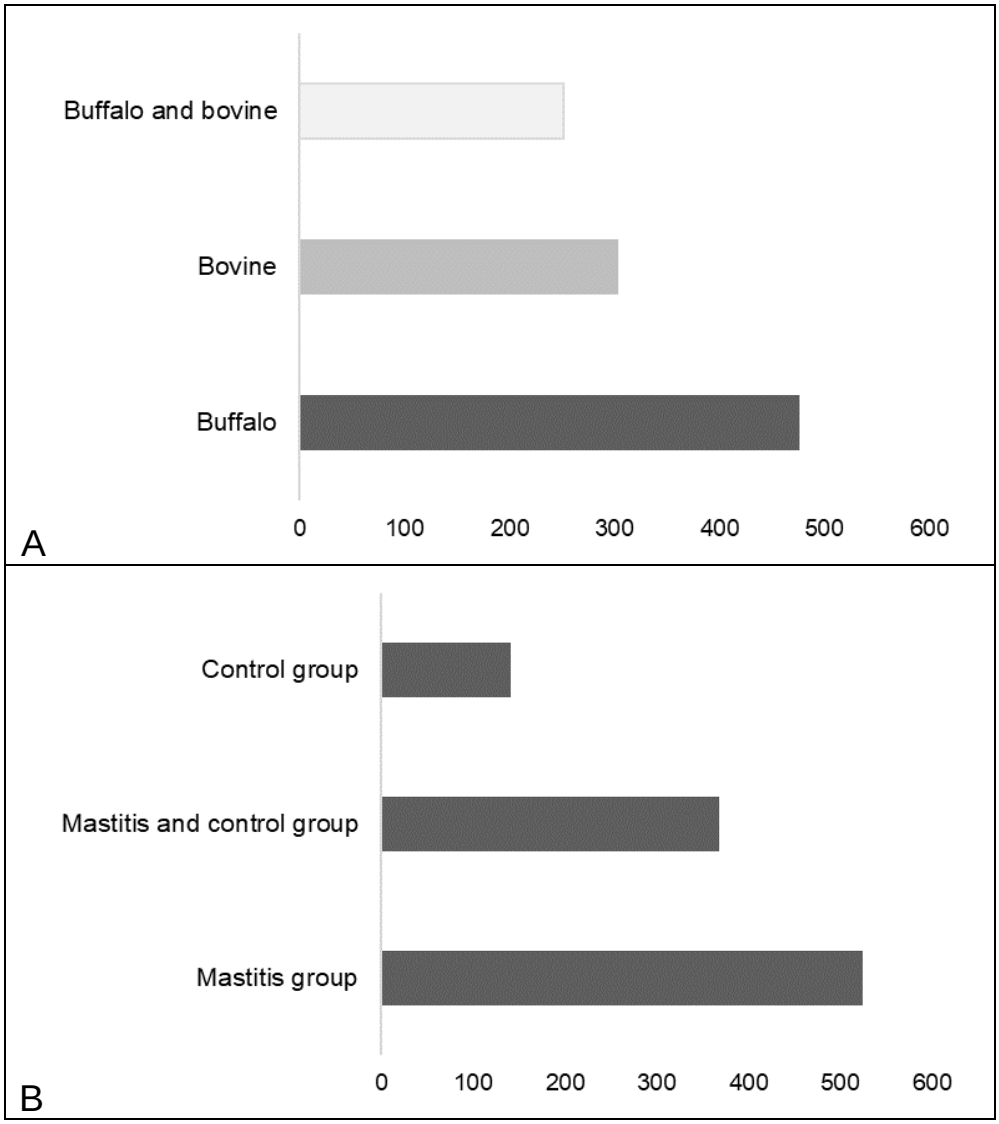


Figure 3: After integration: number of proteins identified for buffalo and bovine reference assemblies (A) and proteins identified in milk whey samples of buffaloes with and without subclinical mastitis using buffalo and bovine reference assembly (B).

The genes responsible for encoding the 1,033 proteins were considered to identify the differentially expressed proteins, using the R/Bioconductor 'msmsTests' package edgeR (negative binomial GLM regression). A total of 156 DEP were found (Table 1), which represent 15% of total proteins identified. The LogFC was used to partition the DEP into up and down-regulated groups with 32 DEP being down-regulated and 124 up-regulated in the mastitis relative to the control group.

Table 1. Differentially expressed proteins identified in the milk of buffaloes with and without mastitis.

Gene symbol	Protein name	LogFC*	p-value	q-value
SERPINB1	Leukocyte elastase inhibitor	6.898	1.03E-22	3.66E-20
LOC102413933	Cathelicidin-2-like	6.787	1.78E-16	3.14E-14
ENO1	Alpha-enolase	6.323	2.86E-13	2.53E-11
CATHL2	Cathelicidin-2	5.899	8.14E-11	4.80E-09
CATH	Cathelicidin-3-like	5.854	2.30E-07	2.91E-06
TKT	Transketolase	5.737	1.27E-09	4.49E-08
CATHL6	Cathelicidin-6	5.726	9.03E-10	3.70E-08
LOC102410742	Alpha-enolase-like, partial	5.671	1.27E-08	2.49E-07
CHI3L1	Chitinase-3-like protein 1	5.665	2.34E-09	6.89E-08
PFN1	Profilin-1	5.605	1.74E-12	1.23E-10
CATHL3	Cathelicidin-3	5.604	6.27E-09	1.31E-07
PGLYRP1	Peptidoglycan recognition protein 1	5.587	9.38E-10	3.70E-08
LOC102414941	Cathelicidin-6-like	5.533	4.66E-09	1.17E-07
CATHL7	Cathelicidin-7	5.482	1.77E-09	5.68E-08
LOC102412295	Cathelicidin-4-like	5.373	2.05E-08	3.62E-07
GPI	Glucose-6-phosphate isomerase	5.365	5.84E-09	1.29E-07
SDS	L-serine dehydratase/L-threonine deaminase	5.318	1.45E-08	2.69E-07
ARHGDIB	Rho GDP-dissociation inhibitor 2	5.272	9.41E-10	3.70E-08
CATHL5	Cathelicidin-5	5.244	6.19E-08	9.53E-07
TMSB4X	Thymosin beta-4	5.177	4.96E-09	1.17E-07
LOC515991	Uncharacterized protein	5.081	9.30E-08	1.37E-06
LCP1	Plastin-2	5.042	1.36E-07	1.84E-06
CD177	LOW QUALITY PROTEIN: CD177 antigen	5.007	3.69E-09	1.01E-07
HRG	Histidine-rich glycoprotein	4.996	9.20E-07	9.87E-06
ALDOA	Fructose-bisphosphate aldolase A	4.796	1.41E-07	1.85E-06
LOC102397971	Glucose-6-phosphate isomerase-like	4.742	3.01E-07	3.56E-06
LOC102402381	Cathelicidin-5-like	4.709	5.24E-07	5.99E-06
LOC102413623	Cathelicidin-7-like	4.532	2.36E-05	0.00018
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	4.421	9.73E-07	1.01E-05
ENO3	Beta-enolase	4.395	1.42E-06	1.36E-05
LOC102400550	Thymosin beta-10-like	4.297	3.89E-06	3.44E-05
TMSB10	Thymosin beta-10	4.297	3.89E-06	3.44E-05
CFL1	Cofilin-1	4.237	9.47E-06	7.98E-05
MMP9	LOW QUALITY PROTEIN: matrix metalloproteinase-9	4.123	0.00016	0.001
PNP	Purine nucleoside phosphorylase	4.084	3.05E-05	0.00022
FABP5	Fatty acid-binding protein, epidermal	4.012	4.81E-05	0.00032
LCN2	Neutrophil gelatinase-associated lipocalin isoform X1	3.928	3.31E-08	5.58E-07

LOC102412272	Cofilin-1-like, partial	3.874	5.79E-05	0.00037
ENO2	ENO2 protein	3.864	4.28E-05	0.00029
TPI1	Triosephosphate isomerase	3.852	0.0002	0.00114
LOC102390936	Peptidyl-prolyl cis-trans isomerase A-like	3.811	2.40E-05	0.00018
PPIA	Peptidyl-prolyl cis-trans isomerase A	3.811	2.40E-05	0.00018
LOC102404617	Peptidyl-prolyl cis-trans isomerase A-like	3.787	2.67E-05	0.00019
HP	Haptoglobin	3.776	6.14E-08	9.53E-07
HIST1H1E	HIST1H1E protein	3.726	0.00013	0.00078
LOC102408803	Histone H1.4-like	3.726	0.00013	0.00078
HMGN2	Non-histone chromosomal protein HMG-17	3.725	0.00019	0.00109
LOC102401516	Non-histone chromosomal protein HMG-17-like	3.725	0.00019	0.00109
LOC102391467	15 kDa protein B-like	3.72	0.00041	0.00218
ENSBTAG00000047529	Uncharacterized protein	3.542	3.87E-05	0.00027
SERPINB6	Serpin B6	3.541	0.00025	0.00135
SERPINB8	Serpin B8	3.541	0.00025	0.00135
SERPINI2	SERPINI2 protein	3.541	0.00025	0.00135
APOE	Apolipoprotein E isoform X1	3.515	1.19E-06	1.20E-05
SH3BGRL3	SH3 domain-binding glutamic acid-rich-like protein 3	3.506	0.00047	0.00239
HIST1H1C	Histone H1.2	3.398	0.00057	0.00274
LOC102390145	Histone H1.2-like	3.398	0.00057	0.00274
TAGLN2	Transgelin-2	3.385	0.00096	0.00446
IGHE	Uncharacterized protein	3.252	0.00056	0.00274
LOC102413454	Peptidyl-prolyl cis-trans isomerase A-like	3.241	0.00139	0.00632
ENSBTAG00000009580	Uncharacterized protein	3.157	0.0017	0.00743
HIST1H1A	Histone H1.1	3.065	0.00253	0.00996
HIST1H1D	Histone H1.3	3.065	0.00253	0.00996
LOC102390920	Histone H1.1-like	3.065	0.00253	0.00996
LOC102410341	Histone H1.4-like	3.065	0.00253	0.00996
LOC102412313	Histone H1.3-like	3.065	0.00253	0.00996
TCN1	Transcobalamin-1	3.049	0.00218	0.00942
LOC102400767	Glyceraldehyde-3-phosphate dehydrogenase-like, partial	3.028	0.00265	0.00999
LOC102403908	Glucose-6-phosphate isomerase-like	3.005	0.00342	0.01223
CFL2	Cofilin-2	2.986	0.00362	0.0127
CP	Ceruloplasmin-like	2.907	1.43E-13	1.69E-11
ACTC1	Actin, alpha cardiac muscle 1	2.904	8.79E-07	9.72E-06
HCN1	Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1-like	2.899	0.00574	0.01815
ACTA1	Actin, alpha skeletal muscle	2.85	1.33E-06	1.30E-05

ACTA2	Actin, aortic smooth muscle	2.829	1.36E-05	0.00011
ACTG2	Actin, gamma-enteric smooth muscle	2.759	2.20E-05	0.00017
SAA3	Serum amyloid A protein	2.752	0.0015	0.00674
LOC104968478	Serum amyloid A protein	2.725	0.00046	0.00235
A2M	Alpha-2-macroglobulin	2.688	2.62E-07	3.19E-06
SAA1	Serum amyloid A protein	2.626	0.00259	0.00999
ACTR3	Actin-related protein 3	2.588	0.01208	0.0334
LOC102414960	Actin-related protein 3-like	2.588	0.01208	0.0334
M-SAA3.2	Serum amyloid A protein	2.582	0.00455	0.01517
LOC102409057	Serum amyloid A protein-like isoform X1	2.542	0.0038	0.01293
ACTB	Actin, cytoplasmic 1	2.503	9.93E-08	1.41E-06
LOC102410151	Serum amyloid A protein-like	2.494	0.00366	0.0127
LOC102391101	Thioredoxin-like	2.472	0.01031	0.02931
TXN	Thioredoxin	2.472	0.01031	0.02931
ACTBL2 - isoform 1	Beta-actin-like protein 2 isoform X1	2.436	2.72E-06	2.54E-05
ACTR3B	Actin-related protein 3B-like	2.404	0.01975	0.04629
HSPA8	Heat shock cognate 71 kDa protein	2.397	0.00605	0.01895
CRISP3	Cysteine-rich secretory protein 3	2.365	0.00976	0.02833
LOC102416626	Complement factor H-like, partial	2.31	0.00674	0.02075
C4	Complement C4	2.248	0.00296	0.01104
ITIH4	Inter-alpha-trypsin inhibitor heavy chain H4	2.232	0.00048	0.00241
LOC617696	Uncharacterized protein	2.189	0.00518	0.01666
ACTG1	Actin, cytoplasmic 2	2.169	1.50E-05	0.00012
LDHA	L-lactate dehydrogenase A chain	2.145	0.00834	0.02481
EZR	Ezrin	2.139	0.00627	0.01946
LOC102414975	Heat shock cognate 71 kDa protein-like	2.095	0.01589	0.03907
LOC102390213	Putative V-set and immunoglobulin domain-containing-like protein IGHV4OR15-8-like, partial	2.028	0.02102	0.04825
LOC102398391	Putative V-set and immunoglobulin domain-containing-like protein IGHV4OR15-8-like, partial	2.028	0.02102	0.04825
LOC102403576	Putative V-set and immunoglobulin domain-containing-like protein IGHV4OR15-8-like, partial	2.028	0.02102	0.04825
JCHAIN	Immunoglobulin J chain	2.017	0.00119	0.00547
MSN	Moesin	2.009	0.01245	0.03415
RDX	Radixin	1.967	0.015	0.03772
LDHB	L-lactate dehydrogenase B chain	1.96	0.00042	0.00222
IGJ	Immunoglobulin J chain	1.897	0.00241	0.00996

FGB	Fibrinogen beta chain, partial	1.808	0.00944	0.02761
LOC102412066	L-lactate dehydrogenase B chain-like, partial	1.738	0.01784	0.04297
ACTBL2 - isoform 2	Beta-actin-like protein 2 isoform X2	1.717	0.00302	0.01115
CFB	Complement factor B	1.684	4.87E-05	0.00032
CFH	Complement factor H	1.646	0.00775	0.02344
FGA	Fibrinogen alpha chain	1.573	0.00223	0.00951
LOC102416562	Fibrinogen gamma-B chain-like	1.554	0.00459	0.01517
LOC100297192	Uncharacterized protein	1.328	0.00376	0.01291
ENSBTAG00000047986	Uncharacterized protein	1.309	0.00449	0.01513
IGLL1	Uncharacterized protein	1.171	0.00244	0.00996
LTF	Lactotransferrin-like	1.072	0.00321	0.01171
LOC102395699	Alpha-S2-casein-like	1.062	0.00485	0.01574
CLU	Clusterin	1.05	4.92E-06	4.25E-05
ENSBTAG00000039237	Uncharacterized protein	0.974	0.00545	0.01738
HPX	Hemopexin	0.903	0.01884	0.04477
LOC102413388	Clusterin-like, partial	0.718	0.00355	0.01257
LOC102406615	Beta-lactoglobulin-like isoform X1	-0.381	0.00464	0.0152
LGB	Beta-lactoglobulin	-0.407	0.01035	0.02931
PAEP	Uncharacterized protein	-0.462	0.01967	0.04629
LALBA	Alpha-lactalbumin	-0.589	0.00044	0.0023
LPO	Lactoperoxidase	-0.74	0.02142	0.04861
KRT9	Keratin, type I cytoskeletal 9	-1.021	0.02113	0.04825
NUCB2	Nucleobindin-2 isoform X1	-1.17	0.0082	0.02459
APOA4	Apolipoprotein A-IV	-1.411	0.01744	0.04257
SELENOM	LOW QUALITY PROTEIN: selenoprotein M-like	-1.601	0.0075	0.0229
KRT10	Keratin, type I cytoskeletal 10	-1.95	0.00342	0.01223
KRT18	Keratin, type I cytoskeletal 18	-2.075	0.01524	0.03772
KRT24	Keratin, type I cytoskeletal 24	-2.075	0.01524	0.03772
KRT31	Keratin 31	-2.075	0.01524	0.03772
KRT33A	Uncharacterized protein	-2.075	0.01524	0.03772
KRT33B	Keratin, type I cuticular Ha3-II	-2.075	0.01524	0.03772
KRT34	Uncharacterized protein	-2.075	0.01524	0.03772
KRT35	Keratin, type I cuticular Ha5	-2.075	0.01524	0.03772
KRT36	Keratin, type I cuticular Ha6	-2.075	0.01524	0.03772
KRT37	LOW QUALITY PROTEIN: keratin, type I cuticular Ha7	-2.075	0.01524	0.03772
KRT38	Keratin, type I cuticular Ha8	-2.075	0.01524	0.03772
KRT40	Keratin, type I cytoskeletal 40	-2.075	0.01524	0.03772
LOC102389131	Keratin, type I microfibrillar 48 kDa, component 8C-1-like	-2.075	0.01524	0.03772
LOC102389471	Keratin, type I microfibrillar 48 kDa, component 8C-1-like	-2.075	0.01524	0.03772
PLBD2	Putative phospholipase B-like 2	-2.175	0.017834	0.04297
KRT13	Keratin, type I cytoskeletal 13	-2.197	0.00265	0.00999

KRT15	Keratin, type I cytoskeletal 15	-2.197	0.00265	0.00999
CD109	Uncharacterized protein	-2.221	0.01815	0.0434
LAMB1	Laminin subunit beta-1	-2.46	0.01169	0.03285
KRT76	Keratin, type II cytoskeletal 2 oral	-2.513	0.00863	0.02545
TPP1	Tripeptidyl-peptidase 1	-2.83	0.00017	0.00101
LOC102410572	CD109 antigen-like, partial	-2.846	0.0016	0.00706
SIL1	Nucleotide exchange factor SIL1	-2.983	0.00091	0.00429

* log2(fold change), fold change estimates are relative to mastitis group

The genes symbol of 156 DEP were analyzed to find the biological processes (Table 2), their networks (Figure 5) and genes/proteins key. A total of 80 biological processes annotated with Gene Ontology terms were identified, of which only the 18 parental categories were considered.

Table 2. Gene Ontology (GO) terms identified using the Gene Ontology Consortium powered by PANTHER Classification System

GO biological process	Fold Enrichment	P-value
1.Mesenchyme migration (GO:0090131) Genes: *ACTC1, ACTG2, ACTA1, ACTA2*	> 100	1.10E-04
2.Positive regulation of protein localization to early endosome (GO:1902966) Genes: *EZR, RDX, MSN*	> 100	9.26E-03
3.Positive regulation of early endosome to late endosome transport (GO:2000643) Genes: *EZR, RDX, MSN*	99.44	3.10E-02
4.Histone H3-K27 trimethylation (GO:0098532) Genes: *HIST1H1C, HIST1H1D, HIST1H1E*	99.44	3.10E-02
5.Skeletal muscle thin filament assembly (GO:0030240) Genes: *ACTC1, ACTA1, ACTA2*	99.44	3.10E-02
6.Antibacterial humoral response (GO:0019731) Genes: *IGJ, LPO, FGB, LTF*	61.19	4.87E-03
7.Acute-phase response (GO:0006953) Genes: *HP, ITIH4, SAA3, LOC104968478, SAA1*	45.2	8.17E-04
8.Glycolytic process (GO:0006096) Genes: *GAPDH, ENO3, ENO2, ENO1, TPI1, ALDOA, GPI*	44.91	2.26E-06
9.Modulation by host of viral process (GO:0044788) Genes: *APOE, HSPA8, CFL1, LTF*	37.88	3.21E-02
10.Defense response to fungus (GO:0050832) Genes: *CATHL6, PGLYRP1, HRG, LTF*	37.88	3.21E-02
11.Killing of cells of other organism (GO:0031640) Genes: *CATHL6, PGLYRP1, HRG, LTF*	34.59	4.59E-02

12.Protein activation cascade (GO:0072376) Genes: *CFB, C4, CFH, IGHE, FGB*	21.16	3.29E-02
13.Glucose metabolic process (GO:0006006) Genes: *GAPDH, FABP5, SDS, ENO1, TPI1, GPI*	18.65	7.31E-03
14.Single organismal cell-cell adhesion (GO:0016337) Genes: *ACTG1, EZR, SERPINB8, LAMB1, APOA4, MSN, ACTB, FGB*	8.79	2.98E-02
15.Negative regulation of endopeptidase activity (GO:0010951) Genes: *MMP9, SERPINB6, SERPINB8, A2M, ITIH4, SERPINI2, CD109, HRG, LTF, SERPINB1*	8.72	1.90E-03
16.Regulation of protein complex assembly (GO:0043254) Genes: *APOE, LCP1, RDX, PFN1, IGJ, ACTR3, MSN, CLU, ACTR3B, HRG*	6.46	2.77E-02
17.Regulation of anatomical structure size (GO:0090066) Genes: *FGA, CFB, C4, LCN2, CFH, IGJ, APOA4, PGLYRP1, IGHF, FGB, LTF*	6.15	1.42E-02
18.Innate immune response (GO:0045087) Genes: *KRT40, HMGN2, KRT35, NUCB2, KRT33A, KRT24, KRT31, KRT34, KRT15, CD177, CRISP3*	5.99	1.80E-02

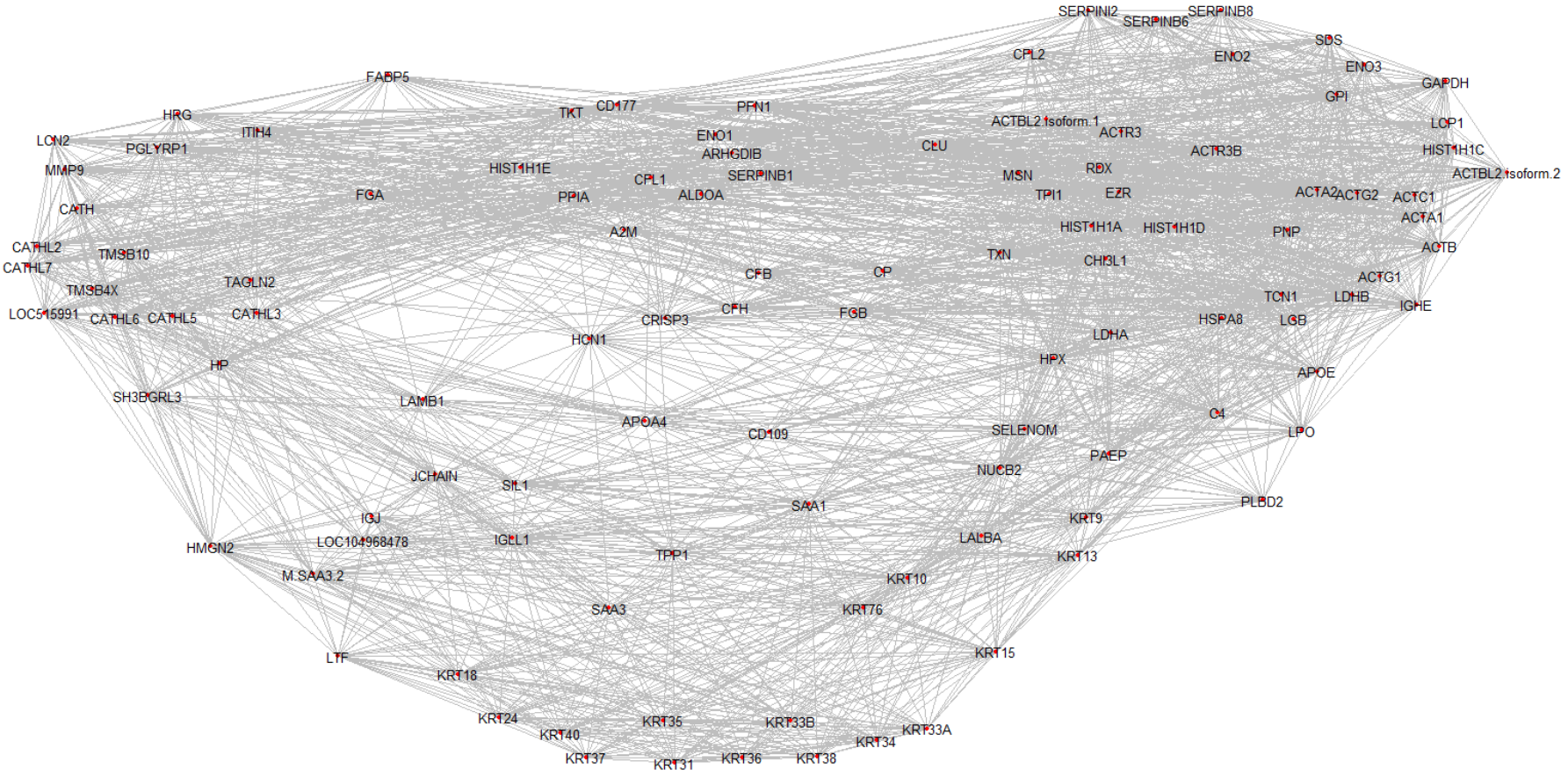


Figure 4: Network analysis of genes responsible for encoding the differentially expressed proteins in milk whey of buffaloes with and without mastitis, using the R/Bioconductor MINET package.

The three proteins considered as the most central group of genes/proteins in the network were CATHL3 (cathelicidin-3), CHI3L1 (chitinase-3-like protein 1) and KRT18 (keratin, type I cytoskeletal 18). Cathelicidin-3 and chitinase-3-like protein 1 were present only in animals with mastitis and were found to be up-regulated in those animals. Conversely, keratin, type I cytoskeletal 18 was present in both groups but was down-regulated in the mastitis group. Assuming the distinction of the counts between the control and mastitis groups and considering the standard error of the mean and standard deviation of these groups, cathelicidin-3 was identified as a potential biomarker of subclinical mastitis.

8. Discussion

In the present study, we considered gene annotations from both buffalo and bovine reference assemblies, due to the fact that the buffalo genome is not fully annotated while that of bovine is better characterized. We used a gene annotation strategy to integrate the annotated proteins, in light of the importance of the use of bovine reference mentioned above. Duplicate gene annotations present for buffalo and bovine reference assemblies were eliminated in favor of the buffalo annotation, and the total number of proteins after integration was 1,033, of which 477 were exclusive to buffalo. We observed the highest number of exclusively identified proteins in the mastitis group and the lowest number identified exclusively in the control group, both before and after integration. This approach can be considered an alternative to software-free analysis, considering the maximum parsimony principle.

Of the total 156 DEP, 64 were found exclusively in buffaloes with mastitis. This number of proteins unique to mastitis shows the change in the protein profiles in milk from animals affected by disease. The three proteins with the highest values of LogFC were leukocyte elastase inhibitor (*SERPINB1*), cathelicidin-2-like (*LOC102413933*) and Alpha-enolase (*ENO1*). The *SERPINB1* gene encodes the leukocyte elastase inhibitor protein, which belongs to a family of serine proteinase inhibitors (serpins), and is involved in the regulation of several biological processes such as blood coagulation, complement activation, inflammation, extracellular matrix remodeling, and tumor suppression [24]. The *ENO1* gene encodes alpha-enolase which is a glycolytic enzyme related to the invasion of host tissue by pathogens [25],

it was first described as a heat shock protein [26] and hypoxic stress protein [27]. The *LOC102413933* gene encodes a cathelicidin-2-like protein, a key protein of the mammalian innate immune defense. These three proteins are, as described above, directly or indirectly related with the defense mechanism.

The LogFC was used to partition the DEP into up and down-regulated groups. A total of 32 DEP were identified as down-regulated and 124 were identified as up-regulated in the mastitis relative to the control groups. Surprisingly, we observed that all differentially expressed cathelicidins (six) were up-regulated and all keratins (sixteen) were down-regulated. The cathelicidin-3 (*CATHL3*) and keratin, type I cytoskeletal 18 (KRT18) were also identified as potential biomarkers, and the importance and functions of these proteins are described below.

The biological processes annotated with Gene Ontology terms showed several functions related with the immune system and metabolic processes (Table 2). The identification of functions such as glycolytic process, mesenchymal migration and glucose metabolic processes suggest that mastitis is affecting the normal metabolic function of dairy buffaloes. Several functions and proteins, such as lactoferrin, related to the defense mechanism were already expected. Lactoferrin is present in six biological processes to date and is the protein that most participates in these biological processes. The *LTF* gene encodes lactoferrin (Lf), an iron-binding and multifunctional glycoprotein, present in colostrum and milk [28]. This protein plays a key role in antimicrobial, as well as antioxidant, anticarcinogenic, enzymatic, immunomodulatory and anti-inflammatory activities [29,30]. In the present study, lactoferrin was one of the differentially expressed proteins, and appears to play a very important role in the immune response in buffaloes with subclinical mastitis.

As mentioned above, cathelicidin-3 (CATHL3), keratin type I cytoskeletal 18 (*KRT18*), and chitinase-3-like protein 1 (*CHI3L1*) were the proteins considered as the most central group of players in the network. Keratins are fibrous structural proteins and intermediate filament-forming proteins of epithelial cells. These proteins are grouped into two distinct subtypes, the type I and type II keratins [31]. Keratins are the predominant proteins in the teat canal lining in bovine [32], and the *KRT16* was indicated as a regulator of innate immunity in mouse [33]. On the other hand, the presence of keratin on the outer layer of skin, in hair and nails, can often be a source

of contamination in mass spectrometry [34]. In our investigation, the keratin type I cytoskeletal 18 was present in buffaloes with and without mastitis and was identified as down-regulated in the mastitis relative to the control groups, different from cathelicidin-3 and chitinase-3-like protein 1 that were only present in animals with mastitis and were identified as up-regulated.

Research with *CHI3L1* in humans suggests that it may be associated with cancer [35,36], and inflammatory diseases [37,38]. Additionally, *CHI3L1* plays a key role in antipathogen response, participating in the innate immune response at the site of pathogen invasion [39]. Associated with the innate immunity, two pro-inflammatory cytokines, *TNF-α* and *IL-1β* genes, also studied in animals affected by mastitis, stimulate the expression of *CHI3L1* in articular chondrocytes [40]. Similar to our study, chitinase-3-like protein 1 was identified only in whey protein from mastitic bovine milk [7].

Although the two proteins described above are related to the immune system, our results indicate that it is cathelicidin-3 that may be a potential biomarker for subclinical mastitis. The cathelicidins comprise a family of antimicrobial peptides (AMPs), that contain a conserved N-terminal sequence (‘cathelin’ domain) and a C-terminal cationic antimicrobial domain [41]. Members of the Cathelicidin family are stored in neutrophil granules as inactive precursors (prepropeptides). After they are cleaved from the holoprotein, C-terminal peptides express antibiotic activity. In humans, they represent a component of the innate immune system [42], and in dairy cows, cathelicidin-1, cathelicidin-2, cathelicidin-3, and cathelicidin-4 were reported in mastitic milk [43]. Cathelicidin-1 was previously described as a biomarker for the detection of subclinical mastitis in bovine [44]. According to Smolenski et al. [45] cathelicidin levels increase in milk following bovine mastitis and monitoring the levels of cathelicidin-based biomarkers through in-line antibody based sensors, may aid in the detection of mastitis.

9. Conclusions

The alteration in the buffalo milk whey fraction caused by subclinical mastitis has been demonstrated by the number of proteins unique to affected animals. A total

of 156 differentially expressed proteins were identified in the milk whey of buffaloes with and without subclinical mastitis, of which cathelicidin-3 can be considered a potential biomarker of subclinical mastitis. These results are important for the study of buffalo mastitis and the identification of cathelicidin-3 may aid in developing strategies for early diagnosis of the disease.

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