Abstract: The objective is to study the effects of nutrient restriction inducing a metabolic imbalance on the inflammatory response of the mammary gland in early lactation cows. The aim is to decipher involved molecular mechanisms by comparing a control and a restriction group transcriptome and proteome after an intramammary lipopolysaccharide challenge. Multiparous cows were either allowed ad libitum intake of a lactation diet (n = 8), or a ration containing low nutrient density (n = 8; 48% barley straw and dry matter basis) for four days starting at 24 ± 3 days in milk. Three days after initiation of treatments, one healthy rear mammary quarter was challenged with 50 µg of lipopolysaccharide (LPS). Transcriptomic and proteomic analyses were performed on mammary biopsies obtained 24 hours after the LPS challenge, using bovine 44K microarrays and nano-LC-MS/MS, respectively. As expected, restriction induced a marked negative energy balance (41 versus 97 ± 15% of Net Energy for Lactation (NEL) requirements) and metabolic imbalance. Microarray analyses identified 25 differentially expressed genes in response to restriction, suggesting that restriction modified mammary metabolism, specifically β-oxidation. Proteomic analyses identified 53 differentially expressed proteins, and suggest modifications of protein synthesis from mRNA splicing to folding. Undernutrition influenced mammary gland expression of genes involved in metabolism, increasing β-oxidation and altering protein synthesis, which may affect the response to inflammation.

Keywords: nutrigenomics; transcriptomic analysis; proteomic analysis; mammary gland; cows; inflammation

1. Introduction

Milk is synthesized in mammary glands (MG) involving a large number of genes, the expression of which is modulated at a nutritional level [1] and by the health status [2]. Mastitis is the inflammatory response of the mammary gland to pathogens. This pathology is the major and costliest problem in dairy cattle worldwide due to decreased milk production, discarded milk, cost of veterinary services and culling [3]. Mastitis is caused by various microorganisms (bacteria, fungus,
and viruses). Gram-positive bacteria, such as *Staphylococcus aureus* and *Streptococcus uberis* might cause persistent infections, with pathogens surviving inside host cells [4,5], whereas Gram-negative coliform bacteria such as *Escherichia coli* most frequently cause an acute inflammation and, eventually, severe mastitis with clinical signs [6]. Cows are particularly susceptible to *E. coli* MG inflammation during the periparturient period due to altered immune function [7]. Early lactation is often associated with metabolic disorders related to stress, energy deficit and mobilization of the body reserves, hypocalcemia, and metritis, which are likely to influence immune function [8,9,10]. Negative energy balance (NEB) affects the inflammatory response, which could be due to changes in the metabolic milieu such as the increased concentration of circulating ketone bodies [8,11]. Undernutrition, however, had minor effects on the response to lipopolysaccharide (LPS) and *S. uberis* challenges in mid-lactation dairy cows [12,13]. The current study is conducted during early lactation Holstein cows, which is characterized by enhanced metabolic deviations in response to NEB that might influence immune system function.

Transcriptome and proteome profiling techniques are available to study inflammation-related changes and enhance the understanding of host–pathogen interactions. Previous research has employed reproducible protocols to challenge lactating cow udders with live pathogens such as *E. coli*, *S. aureus* or using LPS to induce an acute inflammatory response [12,13,14,15]. High-throughput gene expression technologies as transcriptomic analyses have been used to decipher the molecular mechanisms of MG response to inflammation [16] showing that LPS is a strong stimulator of the expression of genes of an inflammatory response [17]. Transcriptomic analyses showed that MG differentially expressed genes (DEG) 24 hours after *E. coli* infection in early [18] and mid [19]–lactating cows. Furthermore, hierarchical clustering of DEG, showed a sharp separation of the infected and the control group [19]. Similar results were observed in primary bovine mammary epithelial cells after challenging of *E. coli* or *S. aureus* [20,21,22]. Modifications of bovine milk proteome were reported during inflammation [23,24]. To our knowledge, the effects of negative energy balance on the responses to acute inflammation has not been studied at protein expression level in MG of early lactation cows.

We hypothesized that aggravated undernutrition in early lactating cows would modify the inflammatory response at mRNA and protein levels. Thus, the objective of this study is to evaluate the effects of undernutrition and the resulting metabolic imbalance on MG transcriptome and proteome in early lactation Holsteins challenged with intramammary LPS. The effects of LPS challenge are already well described in the literature, thus attention is focused on the effects of restriction during inflammation by comparisons of MG transcriptome and proteome of control versus underfed early lactating cows.

2. Results

2.1. Dietary and inflammatory challenges influenced milk and blood composition

Prior to diet treatments at 24 ± 3 days in milk (DIM), no differences were observed for intake, milk yield, composition and component yield, Net Energy for Lactation (NEL) balance, body score (BS), body weight (BW), plasma metabolite and insulin concentrations. Feeding the ration containing 48% of straw (restricted group: REST) induced an immediate depression of dry matter intake (DMI) and decreased energy balance from 5.2 ± 8.9 to -67.2 ± 18.9 MJ/day one day before (corresponding to day 23) and on the last day of restriction (day 27), respectively. Plasma concentrations of glucose and insulin decreased, whereas non-esterified fatty acids (NEFA) and beta-hydroxy butyrate (BHBA) increased dramatically in REST (Supplementary Table S1). The 96 hours of nutrient restriction decreased milk yield from 37.9 to 22.4 kg/d (p<0.001) and milk protein yield from 1.12 to 0.62 kg/day (p<0.005) in REST. These variables were unchanged in control (CONT) cows [25]. Within 2 to 6 h hours after injection with lipopolysaccharide (LPS) we noticed the edema of the challenged quarter, an increase in rectal temperature up to 39.5 °C (temperature increment was +2.1 ± 0.15 °C). The effect of inflammation also was confirmed by milk somatic cell count (SCC). The day before LPS challenge, whole udder composite milk (from PM and AM milking) SCC was 78 000/mL and 92 000 /mL (P =
The SCC response was greater in REST compared to CONT cows (6919 versus 1956 × 1000 per mL, respectively) in composite milk samples from the two milkings that followed LPS injection. SCC returned to pre-LPS counts within less than 7 days post LPS challenge and biopsy. Moreover, quarter milk IL-8, IL1-β, TNF-alpha and CXCL3 at time zero (before LPS challenge) did not differ between CONT and REST. Their concentration increased in response to LPS. This data shows that indicators of mammary inflammation did not differ between CONT and REST before the challenge. Further details about animal performance responses are reported elsewhere [25].

2.2. RT-qPCR analysis

RT-qPCR analysis was performed to quantify candidate genes (CCL5, LAP, RBP4, IL8, IL1, STAT3, CD36, and TAP) chosen on the basis of their implication in inflammation [26] Expression of these genes in mammary gland (MG) did not differ between REST and CONT (p≥0.1) 24 hours after the LPS challenge, except for the defensin Tracheal Antimicrobial Peptide (TAP) gene which tended to decrease in REST (p = 0.07). The expression of INSIG1, and CSN2 genes, which are involved in the biosynthesis of milk components and linked to MG metabolism did not differ between CONT and REST (Figure 1).

Figure 1. Effects of nutrient restriction and LPS challenge on gene mRNA expression quantified by RT-qPCR and presented as ΔCT. Comparison of the gene expression does not show a difference between control (CONT; n = 6) and restricted (REST; n = 6) Holstein cows (p≥0.1). The expression of the TAP gene tended to differ (p = 0.07). UXT2, CLN3, and EIF3K were used as housekeeping genes.

2.3. Microarray analysis

Mammary gene expression analyzed by a microarray assay allowed the identification of 33 differentially expressed genes (DEG), including 25 known genes (corrected p<0.05), between CONT and REST, 24 hours after the inflammatory challenge by LPS (Table 1). The expression increased for 19 and decreased for 6 genes in REST compared with CONT. All these DEG presented a fold change (FC) greater than 1.4, with two genes (PDK4 and SLC25A34) presenting a FC greater than 4. Gene ontology and function analyses performed using the Panther software revealed that most DEG are involved in metabolism, including regulation of fatty acid (FA) oxidation, glucose, and protein metabolism and in immune response (Figure 2). Bioinformatics analysis was complemented using
Pathway Studio® software. The results obtained were consistent with those from Panther software.

We focused on the most represented functions, in particular, those involved in metabolism and immune response. We identified DEG involved in FA and glucose metabolism (CPT1A, PDK4, PFKFB4), carnitine shuttle (SLC25A20, CPT1A, SLC25A34), regulation of cellular ketone metabolic process (PDK4), and the key genes in those processes. A number of genes involved autophagy (PFKFB4, DNNED) and immune function (PGLYRP3, KLF13, PLEKHA2, WC7, TRIB2, CXC7, and MBP) processes also were altered.

Table 1. Differentially expressed genes (alphabetic classification) in MG of early lactation Holstein cows in response to undernutrition and LPS challenge. Transcriptomic comparison of control (CONT; n = 6) and restricted diet (REST; n = 6). Normalized microarray data were analyzed using GeneSpring software and moderated t-tests with Westfall–Young correction. p_adj ≤ 0.05 and ranged from 0.01 to 0.04. FC were >1.4.

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Figure 2. Main biological processes of differentially expressed genes in MG of underfed (REST) versus control (CONT) early lactation cows during an acute inflammation identified by transcriptomic analysis. Bioinformatics analyses were performed using Panther and Pathway Studio® software.

2.4. LC MS/MS proteomic analysis

We identified 1475 proteins, 967 of which were validated with more than two peptides and considered for further investigation. Fifty three proteins were differentially expressed proteins (DEP), 10 were upregulated and 43 were downregulated in REST ($p<0.05$; Table 2; Supplemental File 2). Classification of DEP highlighted proteins involved in the immune process, metabolism (regulation of protein catabolic and carbohydrate metabolic processes) and cell functioning (such as RNA splicing, translation, or regulation of cell adhesion). Proteins involved in apoptosis also are identified (Figure 3). Analysis of DEP using Panther and Pathway Studio® showed that DEP are involved in protein folding and post-translational modifications (GANAB, PDIA3, RPN2, RPN1, CCT4, PDIA4, PPIB), protein catabolic process (PSMD2, PPP2CA), carbohydrate metabolism (PAPSS1, RPS27A, GANAB), synthesis of immunoglobulins (FIMH40, F1MLW8), and regulation of inflammatory response (PDIA3, PSMA3, PSMD2, PPP2CA, RPS2, RPL10, RPS15, CASP6, PCBP2, STAT5A). Many DEP are involved in RNA splicing (HNRNPH1, DHX9, HNRNPC, YBX1, PCBP2, SNRPA1, HNRNPA3, PPP2CA) or translation processes (RPS27A, RPL10, RPS15, RPS2, EIF3H, RPN2, RPN1, FARSB, NACA). Additional analysis by Uniprot software revealed similar classifications confirming them.

Table 2. The list of differentially expressed proteins (up- and down-regulated then alphabetic classification) 24 hours after inflammation challenge by LPS in MG of underfed (REST; n = 6) compared with control (CONT; n = 6) cows. Proteins were analyzed with Progenesis LC-MS software v4.1 (Nonlinear Dynamics). The minimum mascot score validation for one peptide was 31 with a rate of false discovery <1%.

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Figure 3. Main biological processes of differentially expressed proteins in MG of underfed (REST) versus control (CONT) early lactation cows during acute inflammation. Bioinformatics analyses were performed using Uniprot and Pathway Studio® software.

3. Discussion

This study assesses the effects of undernutrition and the resulting metabolic imbalance on the mammary gland (MG) inflammatory response in early lactation cows using a nutrigenomic approach. The effects of dietary treatments are confirmed by decreased intake, milk yield, energy balance in underfed (REST) cows, and by changes in blood metabolite and insulin concentrations (an increase in plasma NEFA and BHBA and a decrease in insulin and glucose concentrations; Supplementary Table S1; [25]). The inflammatory response to intramammary lipopolysaccharide (LPS) is confirmed by clinical parameters such as milk SCC, rectal temperature and other classical clinical symptoms [25]. The effects of nutrient restriction and metabolic imbalance on the inflammatory response at the RNA and protein levels were evaluated using transcriptomic and proteomic analyses. These analyses were performed using MG samples obtained by biopsies performed 24 hours after LPS challenge. We performed a single biopsy to avoid the potential interference of repetitive biopsies on inflammation response. Also, the adjacent quarter may not constitute a good control for the LPS-challenged quarter, as inflammation cytokines may exert local effects and influence adjacent quarters [19]. However, one limitation of this design is that the present experimental design does not allow a kinetic data to follow the establishment of inflammation.

3.1. Gene expression changes at mRNA level

Prior to undertaking a global analysis at mRNA level, we investigated the effects on candidate genes by RT-qPCR, the majority which involved in immune response of interest for inflammation. The expression of candidate genes did not differ between REST and with the control (CONT) group (Figure 1). This result suggests that the expression of genes considered important in the inflammatory response [11,26,27,28] are not altered by the nutrient restriction in MG 24 hours after LPS administration. This experimental design does not allow to evaluate the modification of the expression of these genes during early inflammatory response. A tendency for decreased expression of the TAP gene in REST was observed. The product of TAP gene is a member of the family of small cationic peptides that have widespread antimicrobial activity; TAP is expressed by bovine mammary epithelial cells [29] and has broad-spectrum activity against different strains of bacteria, including E.
The upregulation of TAP gene expression in REST may constitute a protection mechanism against pathogens.

To complete the candidate gene analyses, a global gene expression approach using a bovine microarray was used to assess the molecular mechanisms underlying metabolic and inflammatory MG responses potentially affected by undernutrition and negative energy balance. Transcriptomic analysis revealed 33 differentially expressed genes (DEG) in MG 24 hours after LPS challenge in REST compared to CONT. The number of DEG detected in our study is small compared to research assessing the effects of inflammation on MG transcriptome [18,19,31]. This study does not compare normal versus inflamed MG, rather it aims to evaluate the effects of undernutrition on inflammation response, and therefore both REST and CONT were challenged with LPS. The DEG were classified in six functional classes. In this discussion, we mainly focus on genes that play a role in metabolic processes (FA, glucose and protein metabolism) and that may modulate immune function.

3.1.1. DEG involved in metabolism

The classification of genes by bioinformatics analyses indicate that metabolic process (FA oxidation, glucose and protein metabolisms) is the class most altered by undernutrition after an LPS challenge conditions. Among the genes presenting the highest fold change (between 2.7–6.8) are PDK4, CPT1, and SLC25A34, which are involved in glucose and FA metabolism. PDK4 plays a key role by inhibiting the pyruvate dehydrogenase complex. This inhibition prevents the formation of acetyl-coenzyme A from pyruvate [32], resulting in a decrease in glucose and an increase in fat utilization in response to prolonged undernutrition [33]. Upregulation of PDK4 was also found in leucocytes of underfed ewes, however, its expression was downregulated during an intramammary inflammatory challenge [34]. The large increase of PDK4 expression in REST is in agreement with decreased insulinemia and with the upregulation of ESRRB. The gene ESRRB upregulates PDK4 expression [35]. Both genes spare glucose and promote FA β-oxidation, therefore, their upregulation in REST would allow MG to shift the metabolic pathways from glycolysis to β-oxidation. The upregulation of CPT1 gene expression would also promote the β-oxidation [36,37], since it is rate-limiting step of FA entry in mitochondria [38]. This is in line with the increased expression of the CPT1 gene observed in whole blood transcriptome of underfed dairy sheep [34]. The increase of β-oxidation is further supported by an upregulation of SLC25A20 and SLC25A34 in REST, which are two members of SLC25 mitochondrial carrier family. SLC25A20 transports carnitine and carnitine-FA complexes across the inner mitochondrial membrane. SLC25A34 is supposed to act in a similar way, but its exact function still is not known totally [39]. MG seems to spare glucose (downregulating glycolysis) and promote FAs as an energy source (upregulating β-oxidation; Figure 4) in order to adapt to underfeeding. Mammary expression of genes involved in lipid metabolism also is modified in a comparison between NEB (induced by caloric restriction) and the positive energy balance of cows after the peak in lactation [40]. Interestingly, a downregulation of genes linked to fat metabolism (FA biosynthesis) is observed 24 hours after E. coli infection in MG of lactating cows [18]. This suggests that inflammation downregulates the FA biosynthesis and may increase the use of preformed FA derived from other sources, such as from the mobilization of adipose tissue.

These results suggest that energy metabolism modifications, in response to inflammation, are more marked in REST than CONT, probably due to the limited availability of nutrients to support an acute inflammation in REST.
However, thomeostasis is impaired by undernutrition and the effects of LPS on gene expression show that immune response to infection with different strains of E.coli is not the same as the immune response to LPS stimulus. When the invading bacteria survive, neutrophil infiltration is replaced with T and B lymphocytes and monocytes [43]. The upregulation in REST of KLF13, PLEKH2, WC7, and MBP, involved in the immune response by activating T and B lymphocytes, therefore, is in line with the expect recruitment of leukocytes by MG [Figure 5]. Additionally, the upregulation of PLEKH2 in REST, a gene involved in the cell adhesion process [44], suggests an increased migration of B leucocytes. Together, the upregulation of these genes suggests a different nature or a higher response to LPS stimuli in REST compared with CONT. Nevertheless, the deregulation of TRIB2 and CXCR7 suggests that the immune process could be impaired. Indeed, both genes participate in the activation of immune cells and influence IL-8 production, a chemokine upregulated in response to infection [45] which concentration increased in milk within 4 hours after LPS infusion ([25] and within 16–24 hours after experimental infection with different strains of E.coli or LPS infusion [46,47]. The upregulation of TRIB2 and downregulation of CXCR7 genes, however, suggests a potential IL-8 production alteration in response to inflammation in REST. These results contrast with the expected inflammatory response and could be a sign of deficient immune function under exacerbated NEB. Taken together, differences in gene expression might suggest a modified resolution of inflammation in response to LPS due to aggravated NEB. During the course of an experiment with LPS, the inflammatory response usually declines within 24 hours [46]. The REST cows might have experienced difficulties in restoring the MG homeostasis by 24 hours after LPS challenge due to metabolic changes inherent to nutrient deficiency. However, this conception needs more detailed investigations, with a kinetic analysis, to be confirmed.
3.2. Gene expression at protein level

3.2.1. Proteins involved in protein synthesis

Among the 53 differentially expressed proteins (DEP) in REST compared to CONT, 43 were downregulated. Most of these are involved in RNA and protein metabolism, with roles that vary from RNA splicing to translation. The downregulation of proteins involved in the splicing process, such as HNRNPH1, HNRPC, HNRNPA3, PCBP2, YBX1, SNRPA1, and DHX9, suggests that splicing is impaired in the MG of REST cows. This could explain in part the reduced synthesis and secretion of milk protein in REST compared with CONT [25]. Moreover, altered splicing and translation mechanisms might have a profound influence on protein biochemical properties and, ultimately, alter immune response to pathogens. Among the four proteins belonging to the HRNPs family (HNRNPH1, HNRPC, HNRNPA3, PCBP2), the first three are RNA binding proteins associated with pre-mRNAs in the nucleus, influencing pre-mRNA processing as well as other aspects of mRNA metabolism and transport. The dysfunction of HRNPs is linked to different proliferative and degenerative diseases [48], but the role of these proteins in the inflammatory response is still not fully understood. Some members of this family are reported to ensure resolution of inflammation [49]. In our study, YBX1 and DHX9 are downregulated in REST and the downregulation of these two genes is associated with impaired inflammatory responses [50]. Additionally, the loss of PP2AC function causes severe immunological disorders in Treg cells [51]. Thus, the downregulation of all these proteins in REST (Figure 6) suggest a modified inflammatory response in underfed early lactation cows.

A number of proteins involved in translation are downregulated in REST (Figure 6; RPS27A, RPS15, RPS2, RPL10, EIF3H, RP2N2, RPN1, FARSB, and NACA). Four riboprotein family members (3 RPS and 1 RPL) are part of a ribosome. Interestingly, protein building ribosomes alone are shown to affect the other cell processes outside the ribosome like development, apoptosis, and aging during their altered expression levels [52]. Additionally, the decrease of RPN1 and RP2N2, which catalyze cotranslational N-glycosylation, suggests an impaired post-translational protein modification process in the REST group. This process may play an important role in the immune system by creating the glycans on an immune cell’s surface that helps migration of the cell or by glycosylating the various immunoglobulins [53]. Elsewhere, it is reported that this process can be defective during glucose deficit, leading to a reduction of protein glycosylation and harmful accumulation of unfolded proteins.
proteins [54]. The decrease in RPN1 and RPN2 observed in the current study could potentially lead to the creation of misfolded proteins in MG of REST cows.

Additionally, protein folding and its control might be modified in REST due to the downregulation of chaperone proteins such as PDIA3, PDIA4, and CCT4 (Figure 6). PDIA3 and PDIA4 are part of a larger superfamily of a disulfide isomerase family of endoplasmic reticulum proteins that catalyze protein folding [55]. PDIA3 contributes to the correct folding of glycoproteins [56]. The loss of PDI activity and the consequent accumulation of misfolded proteins is associated with chronic inflammation [56,57]. Moreover, PDIA3 is a structural component required for the stable assembly of the peptide-loading complex of the major histocompatibility complex class I pathway. Its activity seems to play a role in lymphocyte T and B function [58]. Added to its role on folding, PDIA4 promotes the immunoglobulin G intermolecular disulfide bonding and antibody assembly in vitro [59]. Because CCT4 assists in the folding of newly translated polypeptides, this function might have been altered in REST [60]. Overall, proteomic data strongly suggest that protein synthesis is impaired by undernutrition at different levels (translation, folding, post-translation modifications).

The modifications of protein metabolism might partially explain the lower milk protein yield from 1.12 to 0.62 kg/observed during restriction.

Figure 6. The role of differentially expressed genes in crucial steps of the control of gene expression. Green boxes indicate genes which are downregulated by nutritional restriction. Light and dark blue represent the nucleus and ribosomes, respectively. Grey boxes represent the endoplasmic reticulum. Differentially expressed proteins between restricted and control cows after four days of undernutrition for restricted cows during LPS challenge.

3.2.2. DEP involved in immune response

Undernutrition downregulated FARSB protein expression. The decrease of this protein is linked with impaired acute inflammation responses in mice [61], suggesting an impaired function of the immune system. In contrast, there was an upregulation of proteins such as SERPINA3, SERPINA3-5, F1MLW8, and Q1RMN8. SERPINA is an acute-phase protein whose concentration can rise during acute and chronic inflammation [62]. F1MLW8 and Q1RMN8 proteins are similar to immunoglobulin lambda and typical for B-cells and are important for its maturation from pre-B cells to mature ones [63]. The increase of these four proteins in REST MG, suggests that the resolution inflammation process was delayed in REST compared to CONT 24 hours after LPS challenge, whereas it could be considered that potentially already it is resolved in CONT. This is in line with the reported peak in SCC at 12 hours that declines 24 hours after LPS challenge [46].

The decreased translation process and post-translational protein modification (folding, glycosylation) that is observed at the protein level might result in altered immune function through an altered synthesis of immune proteins [54]. Thus, this experiment suggests that undernutrition
might slightly impact the inflammatory response of MG to LPS by mainly affecting protein translation, posttranslational modifications, and folding. These might lead to a dysfunctional inflammatory response.

4. Materials and methods

4.1. Ethics statement, treatments and sampling

Cows were housed at the Herbivore Research Unit of INRA Research Center of Auvergne–Rhone–Alpes. Animal procedures were performed in compliance with Regional Animal Care Committee guidelines CEMEAA: Auvergne, French Ministry of Agriculture and European Union guidelines for animal research C2EA-02. All procedures were approved by the regional ethics committee on animal experimentation (APAFIS #2018062913565518). Animals were in their second to the fourth lactation, with body condition score (BCS) was 2.0 to 2.2 (0 to 5 scale) a before feed restricted diet. All animals were observed for uterine disease and did not present any signs of abnormality. Additionally, the health history of each animal was inspected and only those without any health problems within the last 6 months before calving were chosen.

At 24 ± 3 days in milk, sixteen multiparous Holstein cows were either allowed ad libitum intake of a lactation diet CTRL, n = 8, 7.1 MJ/kg DM NEL, 17.4% Crude Protein. This diet was constituted of corn (24.2% dry matter), corn silage (29%), grass silage (25.5%), soybean meal (16.9%) and a complement with vitamins and minerals (0.9%). The underfed (REST) group received a ration diluted with barley straw (48% DM) for 96 h (RES, n = 8; 5.16 MJ/kg DM NEL, 12.2% CP). Therefore, the ratio of forage to concentrate differed from 58.0/42.0 in control (CONT) group to 79.2/20.8 in REST group [25]. Dry matter intake, milk yield, energy balance, plasma insulin, glucose, non-esterified fatty acids (NEFA) and BHB (β-hydroxybutyrate) concentrations did not differ between CONT and REST immediately before underfeeding (21.8, 39.0 kg/day, −5.6 MJ/day, 22 µIU/mL, 3.78, 0.415 and 0.66 mM, respectively, at day −1), but were significantly altered in REST at 72 h of underfeeding (Supplementary Table S1). Following 72 h of restriction or control diet, one healthy rear mammary quarter was injected with 50 µg of lipopolysaccharide (LPS) E. coli 0111:B4; (LPS-EB Ultrapure, InvivoGen, San Diego, CA) diluted in 10 mL of sterile saline (CDM Lavoisier, Paris, France) containing 0.5 mg/mL BSA cell culture grade, endotoxin free, A9576, (Sigma–Aldrich, St. Louis, MO), using a sterile disposable syringe fitted with a sterile teat cannula. Mammary biopsies were performed 24 hours after the LPS injection, as previously described [64], corresponding to 96 h of feed restriction or control diet. Tissue samples were immediately frozen in liquid nitrogen and stored at −80 ºC prior to RNA and protein analyses.

Throughout the study, milk samples were collected at 4 consecutive milkings each week and analyzed for SCC. Only healthy cows were included in the study. Additionally, cows were screened for mastitis one week before and immediately before the LPS challenge using the California Mastitis Test (Neodis, Rambouillet, France) for all quarters, and somatic cell counts of rear quarter milk samples (Galilait, Theix, 63122 Saint Genè–Champanelle, France), one week before and immediately prior to the LPS challenge. Only cows with SCC lower than 100 000 cells/mL in a rear quarter were included in the study. Indeed, cows were considered healthy if quarter SCC was inferior to 100 000 cells/mL and were free of any other signs of health problems [25]. Additionally, foremilk samples were collected from the LPS challenged quarters immediately before the morning milking that preceded the LPS injection (time 0), and at 4, 6, 10 and 24 h after LPS injection. These quarter milk samples were analyzed for IL-8, IL1-β, TNF-alpha, and CXCL3 using Elisa [25].

4.2. RNA preparation and analyses

RNA and protein extractions were performed from the same mammary biopsy samples n = 16 animals, (8 CONT and 8 REST). Total RNA was extracted from 50 mg of the mammary gland (MG) by use of mirVana miRNA Isolation Kit (Thermo Fisher Sciences, US). The concentration and purity of RNA were estimated by spectrophotometry NanodropTH, (ND-1000, NanoDrop Technologies Int. J. Mol. Sci. 2018, 19, x FOR PEER REVIEW
LLC, Wilmington, DE, USA) and using the Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA), respectively. Once these validation steps were completed, only 12 cows (6 RES and 6 CTR) were kept for gene expression analyses at mRNA level, which presented a good and uniform quality of the samples for a microarray experiment.

4.3. RT-PCR analyses

Reverse transcription (RT) was performed on 2 μg of total RNA using the “High Capacity RNA to cDNA” kit and following the manufacturer’s recommendations (Applied Biosystems, France) in a final volume of 20 μL. In parallel, negative controls were performed without RNA. Primers are described in Table 3. UXT2, CLN3, and EIF3K were used as housekeeping genes [65]. Real-time quantitative PCR was performed on the StepOnePlus™ PCR System (Applied Biosystems, France) using 5 μL of 50 fold-diluted single-stranded cDNA and the TFPower SYBRGreen PCR Master Mix according to the manufacturer’s instructions (Applied Biosystems, France). Subsequent to an initial denaturing step (95 °C for 10 min), the PCR mixture was subjected to the following two-step cycle, which was repeated 40 times: denaturing for 15 s at 95 °C and annealing and extension for 45 s at 60 or 62 °C. The results were expressed as fold change of Ct values relative to the control using the ΔCt method [66]. The significance was determined using a t-test with p<0.05 considered as significant.

Table 3: Primer sequences and annealing temperatures used in real-time reverse transcription-PCR assays as the size of the amplicons (in bp).

<table>
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<tr>
<th>Gene symbol</th>
<th>Primers pair</th>
<th>Amplicon size (bp)</th>
<th>T °C</th>
<th>Reference</th>
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<td>ACA GAT GTG GCT TGA GCG TG ACT GGG TCT GTG TTT TGC AGG</td>
<td>186</td>
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<td>Bernard et al, 2012</td>
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<td>CLN3</td>
<td>TTC TGA CTC CTT GGA ACA CA CAA CCT GCC CAC CTA TCA GT</td>
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<td>62</td>
<td>Bonnet et al, 2012</td>
</tr>
<tr>
<td>CSN2</td>
<td>CTC AAA CCC CTG TGG TGG TG AAA GGC CTG GAT GGG CAT AT</td>
<td>332</td>
<td>60</td>
<td>Ollier et al, 2007</td>
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<tr>
<td>EIF3K</td>
<td>CCA GCC CCA CCA AGA AGA A TTA TAC CTT CCA GGA GGT CCA TGT</td>
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<td>104</td>
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<td>138</td>
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<td>Zaros et al, 2007</td>
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<td>LAP</td>
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<td>Lopez et al, 2009</td>
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</table>
4.4. Microarray analyses

Microarray analyses were performed on twelve animals (6 RES and 6 CTRL) using 100 ng of total RNA from each MG sample. Total RNA was amplified, fluorescently labeled, and hybridized to the bovine 4 x 44K microarray (Agilent Technologies, Inc. Santa Clara, USA), and all the procedures described below were performed according to the manufacturer instructions (Agilent Technologies, Inc. Santa Clara, USA). Briefly, for each hybridization, total RNA was linearly amplified and labeled with Cy3 using the one-color Low Input Quick Amp Labeling Kit. Then, 1650 ng of Cy3-labeled cRNA was hybridized on the microarrays using the Gene Expression Hyb Kit. Hybridization was performed for 17 h at 65 °C in a rotating hybridization oven at 10 rpm. Following hybridization, all microarrays were washed and scanned using the Agilent Microarray Scanner G2565A. Resulting TIFF images were processed using Feature Extraction software Version 11 to obtain normalized data. Normalized with 75th percentile shift data were analyzed using GeneSpring software. The moderated t-test with Westfall–Young familywise error rate (FWER) correction was applied \[67\]. Differences were considered significant at an adjusted \(p<0.05\). The data are accessible through the GEO series accession number GSE114975. The classification and functional analyses of differentially expressed genes (DEG) were performed using PANTHER \[68\] and confirmed using Pathway Studio® software (Elsevier, Nederland).

4.5. Protein preparation and analyses

Proteins were extracted by homogenizing 80 mg MG tissue (n = 16; 8 RES and 8 CTRL) in 2 mL lysis buffer (8.3 M urea, 2 M thiourea, 2% CHAPS, 1% DDT). Following homogenization, samples were incubated for 5 min at room temperature and centrifuged at 10,000 g for 30 min at 8 °C. Protein concentrations were measured in supernatant with Quick Start Bradford protein assay (BioRad, Marnes–La–Coquette, France), aliquoted and then stored at −20 °C until further preparation. Sample supernatants were mixed with 1 volume of Laemmli buffer and heated at 60 °C for 5 min. Separation by SDS-PAGE (12% acrylamide) was performed using a Mini-Protean II electrophoresis unit (BioRad, Marnes–La–Coquette, France) and 100 µg protein loaded per lane. To concentrate samples, gels were run at 80 V until the dye front reached the bottom of the concentration gel. Gels were stained overnight in Coomassie brilliant blue G-250. Excised lanes were reduced and alkylated before destaining in 25 mM ammonium bicarbonate with acetonitrile (50/50 v/v). Following dehydration with 100% acetonitrile, gel pieces were dried in a Speed Vacuum and samples were preserved at −20 °C until LC MS/MS analysis.

4.6. LC MS/MS analysis

Proteins were hydrolyzed overnight at 37 °C using 800 ng (80 µL) of sequence grade-modified trypsin (Promega, France) per band. Subsequent to extraction by 64 µL of acetonitrile 100% and sonication, peptides were concentrated in a Speed Vacuum and volume was adjusted to 30 µL with an aqueous solution (99.9% H2O, 0.1% TFA). Peptide mixture (2.5 µL) was injected into the nano HPLC Ultimate 3000, (Thermo Fisher Scientific, Courtaboeuf, France) after a preliminary step of desalting and concentration in the pre-column 300 µm x 5 mm, (ThermoFisher, Courtaboeuf, France) for 6 min, and a second step of separation in an analytical C18 column 75 µm, 25 cm, (Pepmap Thermo Fisher Scientific, Courtaboeuf, France ) with a 10–40% gradient (A: 0.1% FA in water, B: 0.1% FA in acetonitrile) at 450 nL/min. The eluate was electrosprayed through the CaptiveSpray ion source into the mass spectrometer QTOF impact II (Bruker, Wissembourg, France) operated in CID Data Dependent mode. Each MS analysis was succeeded by as many MSMS analysis as possible within 3 sec.
4.7. Protein identification and label-free quantitation

The raw files were loaded, at the end of each LC-MS/MS analysis, into the Progenesis QI software. Nonlinear Dynamics, v 4.1 (Newcastle upon Tyne, UK) and label-free quantitation was performed using a proprietary workflow alignment, peak picking, normalization, design set up, quantitation, and protein identification.

Regarding protein identification (Supplementary File S2), Mascot V.2.5, internally licensed version (www.matrixscience.com) was used with uniprot-ref_Bos taurus database 19.840 sequences (07/2015). The following parameters were considered for the searches: peptide mass tolerance was set to 10 ppm; fragment mass tolerance was set to 0.05 Da and a maximum of two missed cleavages was allowed. Variable modifications were methionine oxidation (M), carbamidomethylation (C) of cysteine and Deamidated (NQ). Protein identification was considered valid if at least two peptides with a statistically significant Mascot score were assigned with a False Discovery Rate (FDR) less than 1%.

Concerning label-free quantitation, all unique validated peptides of an identified protein were included, and the total cumulative abundance was calculated by summing the abundances of all unique peptides allocated to the respective protein. Statistical analysis was performed using the “between subject design” and p-values were calculated by an analysis of variance using the normalized abundances across all runs. Differential proteins were conserved for interpretation if the peptides’ individual abundances showed a good correlation with protein abundance. All differential proteins were inspected manually with these correlation criteria. To extract maximum biological information of differentially expressed proteins, PANTHER [68], Pathway Studio® software (Elsevier, Nederland) and UniProt [69] were used.

5. Conclusion

Undernutrition affected multiple aspects of MG function, as demonstrated by modifications of milk secretion, and MG mRNA and protein expression. During this study, expression analyses were performed 24 hours post LPS challenge corresponding to the period of inflammation resolution. The effects of undernutrition on studied candidate genes, known as major genes related to innate immune responses, were weak. Nutrigenomic analyses, however, showed that undernutrition of early lactating cows modified mammary gland metabolism, but relatively few genes involved in immune response were affected. Therefore, transcriptomic and proteomic analyses pointed out modifications of energy metabolism (fatty acid and glucose), and protein metabolism (synthesis and post-translational modification), respectively. Our data suggest that the process of inflammation resolution could be impaired in REST cows.

Supplementary Materials:

Table S1: Plasma insulin and metabolite concentration at the day of biopsy after dietary treatment and response to LPS challenge. Occurring at day 24 ± 3 of lactation, animals were assigned to a control (CONT, n = 8) or restricted (REST, n = 8) group. The REST animals received the ration diluted with barley straw (48% DM) for 4 days when cows from CONT were allowed to continue ad libitum intake of a lactation diet (7.1 MJ/kg DM NEL, 17.4% CP). Occurring on day 3, corresponding to the 27th day of lactation, the rear mammary quarter of animals from both groups was injected with 50 µg of LPS. Mammary biopsies were performed 24 hours after LPS challenge. p<0.01 for all variables.

File S2: Protein analysis report.


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5. Almeida, RA.; Matthews, KR.; Cifrian, E.; Guidry, AJ.; Oliver, SP. Staphylococcus aureus invasion of bovine mammary epithelial cells. J. Dairy Sci. 1996, 70, 1021-1026, DOI:10.3168/jds.S0022-0302(96)76454-8


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Supplementary Table S1. Plasma insulin and metabolite concentration at the day of biopsy after dietary treatment and response to LPS challenge. At the day 24 ± 3 of lactation, animals were assigned to a control (CONT, n = 8) or restricted (REST, n = 8) group. The REST animals received the ration diluted with barley straw (48% DM) for 4 days when cows from CONT were allowed to continue ad libitum intake of a lactation diet (7.1 MJ/kg DM NEL, 17.4% CP). On day 3, corresponding to the 27th day of lactation, rear mammary quarter of animals from both groups was injected with 50 µg of LPS. Mammary biopsies were performed 24 h after LPS challenge. \( p < 0.01 \) for all variables.

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Supplementary File S2: Protein details analysis

ID0228_Expression Protein Details

Data Processing Methods

Peak processing method  Centroided data; resolution = 30000 (FWHM)
Peptide ion detection method  High resolution

Experiment Design

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Proteins

Protein building options

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Average Normalised Abundances

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**Tags**

- Anova p-value ≤ 0.05
- M
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**Description** Uncharacterized protein OS=Bos taurus GN=HSPA4 PE=1 SV=2

**Peptides** 5 (5)
- **Score** 206.71
- **Anova** 1.83e-003
- **Fold** 1.45

Accession 1::A6H7H3_BOVIN

**Description** LOC789567 protein OS=Bos taurus GN=LOC789567 PE=2 SV=1

**Peptides** 2 (2)
- **Score** 100.91
- **Anova** 2.14e-003
- **Fold** 2.54

Accession 1::PSMD2_BOVIN

**Description** 26S proteasome non-ATPase regulatory subunit 2 OS=Bos taurus GN=PSMD2 PE=1 SV=2

**Peptides** 4 (4)
- **Score** 121.33
- **Anova** 2.73e-003
- **Fold** 1.37
Accession 1::YBOX1_BOVIN (+1)

Description  Nuclease-sensitive element-binding protein 1 OS=Bos taurus GN=YBX1 PE=2 SV=3

Peptides 5 (5)
  Score 214.76
  Anova 3.86e-003
  Fold 1.73

Accession 1::PPIB_BOVIN (+1)

Description  Peptidyl-prolyl cis-trans isomerase B OS=Bos taurus GN=PPIB PE=1 SV=4

Peptides 4 (4)
  Score 258.86
  Anova 4.23e-003
  Fold 1.36

Accession 1::F1MLW8_BOVIN

Description  Uncharacterized protein OS=Bos taurus PE=4 SV=2

Peptides 3 (3)
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  Anova 4.75e-003
  Fold 5.49

Accession 1::EIF3H_BOVIN
**Description** Eukaryotic translation initiation factor 3 subunit H OS=Bos taurus GN=EIF3H PE=2 SV=1

**Peptides** 2 (2)
- **Score** 75.15
- **Anova** 5.32e-003
- **Fold** 1.68

**Accession 1::PLBL2_BOVIN**

**Description** Putative phospholipase B-like 2 OS=Bos taurus GN=PLBD2 PE=2 SV=1

**Peptides** 2 (2)
- **Score** 39.97
- **Anova** 9.21e-003
- **Fold** 1.26

**Accession 1::Q3SYT9_BOVIN**

**Description** Poly(RC) binding protein 2 OS=Bos taurus GN=PCBP2 PE=1 SV=1

**Peptides** 3 (3)
- **Score** 177.92
- **Anova** 9.31e-003
- **Fold** 1.18

**Accession 1::ARC1B_BOVIN**

**Description** Actin-related protein 2/3 complex subunit 1B OS=Bos taurus GN=ARPC1B PE=1 SV=4

**Peptides** 2 (2)
- **Score** 80.22
Anova 9.58e-003
Fold 2.20

Accession 1::E1BF20_BOVIN (+2)

**Description**  Uncharacterized protein OS=Bos taurus GN=HNRNPH1 PE=4 SV=2  
**Peptides**  8 (8)  
**Score**  518.75  
**Anova**  9.96e-003  
**Fold**  1.24

Accession 1::PP2AA_BOVIN

**Description**  Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform OS=Bos taurus GN=PPP2CA PE=1 SV=1  
**Peptides**  4 (4)  
**Score**  155.92  
**Anova**  9.97e-003  
**Fold**  1.19

Accession 1::F1N1G7_BOVIN

**Description**  Kinesin-like protein OS=Bos taurus GN=KIF5B PE=1 SV=2  
**Peptides**  2 (2)  
**Score**  91.70  
**Anova**  0.01  
**Fold**  1.28
Accession 1::GBB1_BOVIN

Description  Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 OS=Bos taurus GN=GNB1 PE=1 SV=3
Peptides  4 (4)
  Score 226.38
  Anova 0.02
  Fold 1.26

Accession 1::G3X6L8_BOVIN

Description  Uncharacterized protein OS=Bos taurus GN=NIPSNAP3A PE=4 SV=1
Peptides  2 (2)
  Score 60.63
  Anova 0.02
  Fold 1.60

Accession 1::Q2KJH7_BOVIN

Description  Aldehyde dehydrogenase 18 family, member A1 OS=Bos taurus GN=ALDH18A1 PE=2 SV=1
Peptides  3 (3)
  Score 121.89
  Anova 0.02
  Fold 1.73

Accession 1::A6QP36_BOVIN
Description LMAN2 protein OS=Bos taurus GN=LMAN2 PE=2 SV=1
  Peptides 4 (4)
  Score 140.38
  Anova 0.02
  Fold 1.22

Accession 1::DHX9_BOVIN

Description ATP-dependent RNA helicase A OS=Bos taurus GN=DHX9 PE=2 SV=1
  Peptides 9 (9)
  Score 359.62
  Anova 0.02
  Fold 1.31

Accession 1::Q3SX47_BOVIN

Description Heterogeneous nuclear ribonucleoprotein C (C1/C2) OS=Bos taurus GN=HNRPC PE=2 SV=1
  Peptides 2 (2)
  Score 146.99
  Anova 0.02
  Fold 1.22

Accession 1::A6H788_BOVIN

Description SNRPA1 protein OS=Bos taurus GN=SNRPA1 PE=2 SV=1
  Peptides 2 (2)
  Score 79.16
  Anova 0.02
Accession 1::F1N6Y1_BOVIN

Description Uncharacterized protein OS=Bos taurus GN=GANAB PE=1 SV=1
Peptides 9 (9)
Score 378.04
Anova 0.02
Fold 1.23

Accession 1::E1BKX3_BOVIN

Description Uncharacterized protein OS=Bos taurus GN=MYBBP1A PE=1 SV=1
Peptides 3 (3)
Score 75.11
Anova 0.02
Fold 1.94

Accession 1::TCPD_BOVIN

Description T-complex protein 1 subunit delta OS=Bos taurus GN=CCT4 PE=1 SV=3
Peptides 9 (9)
Score 512.81
Anova 0.02
Fold 1.25

Accession 1::G8JKW7_BOVIN
**Description** Uncharacterized protein OS=Bos taurus GN=SERPINA3 PE=3 SV=1
- **Peptides** 3 (3)
  - **Score** 231.21
  - **Anova** 0.02
  - **Fold** 2.40

**Accession 1::CAZA2_BOVIN**

**Description** F-actin-capping protein subunit alpha-2 OS=Bos taurus GN=CAPZA2 PE=1 SV=3
- **Peptides** 4 (4)
  - **Score** 124.84
  - **Anova** 0.02
  - **Fold** 1.39

**Accession 2::Q1RMN8 (+1)**

**Description** TREMBL:Q1RMN8 (Bos taurus) Similar to Immunoglobulin lambda-like polypeptide 1
- **Peptides** 5 (5)
  - **Score** 543.36
  - **Anova** 0.02
  - **Fold** 1.89

**Accession 1::PDIA4_BOVIN**

**Description** Protein disulfide-isomerase A4 OS=Bos taurus GN=PDIA4 PE=2 SV=1
- **Peptides** 10 (10)
  - **Score** 441.10
Anova 0.02
Fold 1.20

Accession 1::F6PWD5_BOVIN (+3)

Description Uncharacterized protein (Fragment) OS=Bos taurus PE=4 SV=1
Peptides 6 (6)
Score 396.20
Anova 0.03
Fold 1.29

Accession 1::E1BEG2_BOVIN (+1)

Description Uncharacterized protein OS=Bos taurus GN=HNRNPA3 PE=1 SV=2
Peptides 4 (4)
Score 279.23
Anova 0.03
Fold 1.15

Accession 1::F6QE33_BOVIN

Description Uncharacterized protein OS=Bos taurus GN=COPS7A PE=4 SV=1
Peptides 2 (2)
Score 73.82
Anova 0.03
Fold 1.84
Accession 1::SPA35_BOVIN

Description Serpin A3-5 OS=Bos taurus GN=SERPINA3-5 PE=3 SV=1
Peptides 3 (3)
  Score 256.91
  Anova 0.03
  Fold 1.99

Accession 1::RPN2_BOVIN

Description Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2 OS=Bos taurus GN=RPN2 PE=2 SV=1
Peptides 6 (6)
  Score 351.38
  Anova 0.03
  Fold 1.50

Accession 1::A3KN04_BOVIN

Description Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 OS=Bos taurus GN=RPN1 PE=1 SV=1
Peptides 3 (3)
  Score 148.24
  Anova 0.03
  Fold 1.31

Accession 1::CASP6_BOVIN
**Description** Caspase-6 OS=Bos taurus GN=CASP6 PE=2 SV=1

**Peptides** 2 (2)
- **Score** 86.12
- **Anova** 0.03
- **Fold** 1.47

**Accession 1::RS2_BOVIN**

**Description** 40S ribosomal protein S2 OS=Bos taurus GN=RPS2 PE=2 SV=2

**Peptides** 5 (5)
- **Score** 240.00
- **Anova** 0.03
- **Fold** 1.21

**Accession 1::Q3T0J0_BOVIN**

**Description** 3'-phosphoadenosine 5'-phosphosulfate synthase 1 OS=Bos taurus GN=PAPSS1 PE=2 SV=1

**Peptides** 2 (2)
- **Score** 77.32
- **Anova** 0.03
- **Fold** 1.37

**Accession 1::HBB_BOVIN (+4)**

**Description** Hemoglobin subunit beta OS=Bos taurus GN=HBB PE=1 SV=1

**Peptides** 18 (18)
- **Score** 1356.29
- **Anova** 0.03
Accession 1::RL10_BOVIN (+2)

Description 60S ribosomal protein L10 OS=Bos taurus GN=RPL10 PE=2 SV=4

Peptides 3 (3)
Score 153.13
Anova 0.03
Fold 1.86

Accession 1::F2Z4F0_BOVIN

Description Uncharacterized protein OS=Bos taurus GN=ACTR1A PE=1 SV=1

Peptides 4 (4)
Score 97.27
Anova 0.03
Fold 1.34

Accession 1::RS15_BOVIN

Description 40S ribosomal protein S15 OS=Bos taurus GN=RPS15 PE=2 SV=3

Peptides 3 (3)
Score 209.88
Anova 0.03
Fold 1.40

Accession 1::PRDBP_BOVIN
**Description**  Protein kinase C delta-binding protein OS=Bos taurus GN=PRKCDBP PE=2 SV=1

**Peptides**  2 (2)
**Score**  102.84
**Anova**  0.03
**Fold**  1.14

**Accession 1::G3MY19_BOVIN**

**Description**  Uncharacterized protein OS=Bos taurus GN=PDLIM5 PE=4 SV=1

**Peptides**  3 (3)
**Score**  151.45
**Anova**  0.04
**Fold**  1.80

**Accession 1::F1MH40_BOVIN**

**Description**  Uncharacterized protein OS=Bos taurus PE=1 SV=2

**Peptides**  7 (7)
**Score**  542.47
**Anova**  0.04
**Fold**  1.50

**Accession 1::PSA3_BOVIN**

**Description**  Proteasome subunit alpha type-3 OS=Bos taurus GN=PSMA3 PE=1 SV=3

**Peptides**  2 (2)
**Score**  128.32
**Anova**  0.04
Fold 1.21

Accession 1::RS27A_BOVIN (+1)

Description Ubiquitin-40S ribosomal protein S27a OS=Bos taurus GN=RPS27A PE=1 SV=2
Peptides 6 (6)
Score 387.82
Anova 0.04
Fold 1.19

Accession 1::A8E4P2_BOVIN

Description FARSB protein OS=Bos taurus GN=FARSB PE=2 SV=1
Peptides 3 (3)
Score 85.93
Anova 0.04
Fold 1.42

Accession 1::HBA_BOVIN (+1)

Description Hemoglobin subunit alpha OS=Bos taurus GN=HBA PE=1 SV=2
Peptides 8 (8)
Score 408.92
Anova 0.04
Fold 1.31

Accession 1::F1MN61_BOVIN
**Description**  Uncharacterized protein (Fragment) OS=Bos taurus GN=EEA1 PE=1 SV=2

Peptides 2 (2)
- **Score** 85.75
- **Anova** 0.04
- **Fold** 2.00

**Accession 1::NACA_BOVIN**

**Description**  Nascent polypeptide-associated complex subunit alpha OS=Bos taurus GN=NACA PE=1 SV=1

Peptides 4 (4)
- **Score** 274.55
- **Anova** 0.05
- **Fold** 1.21

**Accession 1::STA5A_BOVIN**

**Description**  Signal transducer and activator of transcription 5A OS=Bos taurus GN=STAT5A PE=2 SV=2

Peptides 2 (2)
- **Score** 87.68
- **Anova** 0.05
- **Fold** 1.39

**Accession 1::ARPC2_BOVIN**

**Description**  Actin-related protein 2/3 complex subunit 2 OS=Bos taurus GN=ARPC2 PE=1 SV=1

Peptides 7 (7)
Score 270.30
Anova 0.05
Fold 1.11

Accession 1::PDIA3_BOVIN

Description Protein disulfide-isomerase A3 OS=Bos taurus GN=PDIA3 PE=2 SV=1
Peptides 16 (16)
Score 845.09
Anova 0.09
Fold 1.11

Accession 1::A7E307_BOVIN (+1)

Description DDX17 protein OS=Bos taurus GN=DDX17 PE=1 SV=1
Peptides 10 (10)
Score 478.07
Anova 0.23
Fold 1.08