ERIKA TORIYAMA

PROTEOMIC PROFILES OF *Longissimus dorsi* PIG MUSCLE

Thesis presented to the Animal Science Graduate Program of The Universidade Federal de Viçosa, in partial fulfillment of the requirements for degree of *Doctor Scientiae.*

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A vida é um conjunto de processos e experiências para se atingir um alvo (...). A felicidade está em cada alvo que você alcança e nos processos que você percorre para alcançá-lo...

Dr Celso Charuri

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SUMARY

RESUMO

TORIYAMA, Erika, D.Sc., Universidade Federal de Viçosa, março de 2013. **Perfis proteômicos do músculo *Longissimus dorsi* de suínos.** Orientadora: Simone Eliza Facioni Guimarães. Coorientadores: Maria Cristina Baracat Pereira e Paulo Sávio Lopes.

A análise proteômica do músculo esquelético de suínos foi realizada com a utilização da solução de RNAholder® para comparar a eficiência na preservação de tecido em relação ao método padrão que utiliza nitrogênio líquido. Foram utilizadas amostras de músculos *Longissimus dorsi* provenientes de 3 animais do mesmo grupo genético (Landrace x Large White) com 5 meses de idade e média de peso vivo de 81,9 kg. A técnica utilizada foi a de eletroforese em gel bidimensional (2-DE) e espectrometria de massas. Doze *spots* de proteínas abundantes que não apresentavam diferenças significativas entre os métodos foram selecionados para avaliar a eficiência da ionização no MALDI-TOF/TOF. Todos os *spots* de proteínas puderam ser igualmente identificados. Os resultados demonstraram que a solução de RNAholder® apresentou maior número de proteínas provavelmente devido à melhor separação. Além disso, esta solução permite a identificação de proteínas e pode ser usada com sucesso para preservar os perfis de proteínas nos estudos proteômicos de músculos de suínos. Em outro estudo, as proteínas do músculo *Longissimus dorsi* da linha Comercial e da raça Brasileira Piau foram separadas pela tecnologia de eletroforese em gel bidimensional (2-DE) e espectrometria de massas. Trinta e cinco *spots* de proteínas apresentaram diferenças significativas entre os dois grupos genéticos. Seis *spots* foram mais expressos na raça Piau; dois mais expressos na linha Comercial; três proteínas estavam presentes somente na raça Piau e 24 presentes somente na linha Comercial. As proteínas mais abundantes indicaram diferenças entre os grupos genéticos divergentes quanto às proteínas estruturais, defesa celular e proteínas relacionadas ao estresse. Os resultados sugerem que a raça Piau apresenta um metabolismo oxidativo ao invés de glicolítico, como os da linha Comercial (Landrace x Large White). O uso do RNAholder® permitiu a identificação da proteína, porém novas investigações de perfis proteômicos podem fornecer mais informações nestes grupos genéticos.

ABSTRACT

TORIYAMA, Erika, D.Sc., Universidade Federal de Viçosa, march, 2013. **Proteomic profiles of *Longissimus dorsi* pig muscle.** Adviser: Simone Eliza Facioni Guimarães. Co-advisers: Maria Cristina Baracat Pereira and Paulo Sávio Lopes.

The proteomics of porcine skeletal muscle using RNAholder® solution was performed to compare the efficiency in tissue preservation in relation to the standard Liquid Nitrogen method. Samples from *Longissimus dorsi* muscle from three animals of the same genetic group (Landrace x Large White) at five month of age and average weight 81.9 kg were utilized. The analyses were performed based in the 2-dimensional electrophoresis (2-DE) and mass spectrometry. Twelve abundant protein spots which were not significantly different between the methods were select to evaluate the efficiency of identifying proteins. All the protein spots could be equally identified. The results demonstrated that RNAholder® solution presented higher number of proteins probably due to better separation. Besides, this solution allows the proteins identification and can be successfully used to preserve protein profiles in porcine muscle in proteomic studies. In other study, proteins from the *Longissimus dorsi* muscle from the Commercial line and local Brazilian breed Piau were separated by 2-dimensional electrophoresis (2-DE) technology and mass spectrometry. At the protein level, 35 protein spots were significantly different between the two genetic groups. Six spots were overexpressed in Piau breed; two overexpressed in Commercial line; three protein spots were present only in Piau breed and 24 spots were present only in Commercial line. The protein abundance indicated differences between divergent genetic groups in structural proteins, defense, and stress-related proteins. The results suggested that the local breed Piau have oxidative metabolism instead the glycolytic one observed in Commercial line (Landrace x Large White). The use of RNAholder® allowed the protein identification, but new investigations in proteomics profiles could provide more information in these breeds.

GENERAL INTRODUCTION

Pig production is one of the main activities for meat industry around the world. In Brazil, according to *Associação Brasileira da Indústria Produtora e Exportadora de Carne Suína* (Abipecs, 2013), the marketing of meat is very important to business, placing the country among the four largest producers behind China, the United States and European Union. In 2012, the exportation had grown about 12.6% in comparison with the year of 2011 and are expected an increase of 20% due to the opening market to Japan in the first trimester of 2013.

New researches are made in order to attempt market needs and to unravel the molecular mechanisms involved in the determination of phenotypic traits. Genome researches consider the genes and the non-coded sequences of DNA and RNA to obtain information about the heritability of organisms. However, this information had shown to be insufficient to related genes to its biological functions. The transcriptomic has begun with the aim to study all molecules of RNA, including the non-coding molecules produced from cell transcription. The gene is transcript to mRNA, mRNA is translating to proteins and the proteins compose and build every cell. According to Hollung et al. (2007), while genes remains constant during the lifetime of the animal, the expression of the genes to mRNA and proteins is very dynamic and is regulated by a large numbers of factors such as environmental and processing conditions.

Despite of the great numbers of genome sequencing projects completed, neither genomic or transcriptomic can explain the biological process in a global sense. The research areas have focused in to relate the gene expression to phenotype of the desirable traits. Thus, the proteome has come as large scale study of protein expression, protein-protein interactions or post-translate modifications.

The proteome is the protein expressed by a genome and consists of the total group of proteins expressed at a certain time point (Wilkins et al., 1996). In contrast to the genome, the proteome is continuously changing according to factors influencing on either protein synthesis or degradation. Thus, analyzing the proteome can be viewed as analyzing snap-shots into a system in constant change (Hollung et al., 2007). The proteome can analyze a thousand of proteins in a single trial and allows demonstrating how the cells can dynamically respond to physiological changes in response to environmental changes. Therefore, the primary goal of proteomics is to identify new

and potentially unexpected changes in the expression of proteins, their interactions or modifications as a result of an experimental treatment. In essence, proteomics allows a researcher see the complete picture of cellular functions rather than an action of a particular protein (Lippolis & Reinhardt, 2008).

The proteomic research represents a major challenge due to the heterogeneity and quantitative protein biochemistry (René Lametsch, 2011) and can be seen as a molecular link between the genome and the expression of the functional quality of the meat, being expressed under given environmental conditions and processing (Bendixen, 2005). While the genome contains the information on which genes are available, the proteome contains information on which genes are actually being expressed.

In meat science, proteomics can be considered a relatively new tool. It is related to muscle development, growth and metabolism function beyond the ante and post-mortem (Bouley et al., 2004; Bendixen, 2005). Studies of associations between phenotypic and genotypic characteristics and their relationships with regulatory and biological mechanisms have been targeted by investigators. Besides, the studies of candidate protein markers for meat quality and traceability, have also been investigated (Ametsch et al., 2003; van de Wiel & Zhang, 2007; te Pas et al., 2013).

Different techniques may be used for the study of the proteome. However, none of them is fully capable of representing all proteins of a certain tissue or extract. The efficiency depends on the characteristics of the material, the number of proteins present in sample, expression level, isoeletric point and molecular weight of proteins, hydrophobicity and the purpose of the study. The large number of factors such as the level of protein expression, can complicate the identification of proteins (Lippolis & Reinhardt, 2008). Therefore, the choice of technique depends on the number of proteins to be investigated because hundreds to thousands of proteins can be analyzed in one experiment, but still be a small part of the entire proteome (Hollung et al., 2007).

The classic and most widely used method in meat science is the two-dimensional gel electrophoresis (2-DE). This method is based on the separation of proteins according to their isoelectric point (Isoelectric Focusing – IEF) in the first dimension, followed by separation according to molecular weight in the second dimension (2-DE). The proteins of interest are then identified by mass spectrometry (MS). The combination of 2-DE and MS is called gel-based proteomic and has been shown to be very efficient for the

analysis of muscles by several authors (Hollung et al., 2008; Y. J. Xu et al., 2009; Y. Xu et al., 2012).

Appropriate sample preparation is essential for obtained reliable results in a proteomic analysis. It is advisable to keep sample preparation as simple as possible to avoid protein losses (Hollung, et al., 2007). Rapidly freezing tissue samples in liquid nitrogen is the standard method of tissue preservation avoiding protein modifications and degradations. Despite the success of this method, the study of other sampling methods prior to proteomic analysis has been reported using RNAlater® (Ambion, Applied Biosystems, California, US) as an alternative to reduce problems concerns of handling liquid nitrogen during sampling in other organisms than pig (Mutter et al., 2004; Jiang et al., 2004; Lenchik, et al., 2005; Abbaraju, et al., 2011;). In Brazil, there is a similar product named RNAholder® (Bioagency, São Paulo, SP, Brazil) solution commonly used to evaluate pattern for expression genes (Serão et al., 2011). The RNAholder® solution allows using samples for simultaneous analysis with transcriptome. This stabilization solution is similar to RNAlater® and until now was not used for tissue preservation in proteomic studies of pigs.

Besides the type of extraction used, the detection and the quantification of proteins are key issues for studying the expression level or the amount of protein (Lametsch, 2011). For detection of proteins in 2-DE gels, is commonly used Coomassie blue staining or silver staining, but they have a limited range and can accurately quantify only one set of proteins made up in a linear region of the gel. Recently, protein detection by fluorescence (2-DE-DIGE, *Difference Gel In Eletrophoresis*) has been used due the wide range and high sensitivity of the technique. In this technique, also known as electrophoresis-2D differential gels, it is possible to conduct multiple tests on the same gel, using fluorescent probes for each sample (Hollung et al., 2008; Li et al., 2013; Tonge et al., 2001).

The detection of the modification of a protein is relatively simple using the 2-DE gel separation parameters such as molecular weight and IEF. Thus, if the protein is phosphorylated, the pH value is also changed, since the balance between the positive and negative charges of the ionic groups of the protein will change. So, the protein will migrate differently during isoelectric focusing in the first dimension (René Lametsch, 2011). If the proteins are modified by cleaving, the molecular weight of the resulting

fragments will change differently and migrate in the second dimension. In this case, in the subsequent analysis of mass spectrometry, the modifications can be characterized and the type and site of modification can be identified (Jensen, 2004).

Despite the widespread use and apparent advantages of the separation of complex proteins by two-dimensional gel, limitations and disadvantages still exist in their use. The choice of the extraction method can determine which proteins are extracted and which will not be considered due property of proteins. The proteins have different chemical properties and are localized in different compartments. Some are part of large protein aggregates like myofibrillar proteins, some are localized in membranes and others are enzymes localized in the cytoplasma (Hollung et al., 2007). In addition, proteins with high (> 150 kDa) or low (<10 kDa) molecular weight as well as proteins with isoelectric points of extreme values, particularly basic proteins, are normally not detected by 2-DE gels. Another drawback is that hydrophobic proteins such as membrane proteins and proteins with a high molecular weight, which due to difficult solubilization during extraction and due to precipitation during electrophoresis, do not appear in the gel. For this reason, the technique of 2-DE gels hinders investigations of many muscle structural proteins such as myosin heavy chain, titin and nebulin, since they have a molecular weight above 150 kDa (Lametsch, 2011). Besides these factors, the technique in 2-DE gel presents technical limitations such as time-consuming, labor intensive and need some experience of the researcher to generate reproducible gels quantitative and spatially.

Therefore, the gel-based proteomics as another proteomics techniques have some limitations and the choice of the better method should be carefully considered in the design. A more advanced strategy that results in a large volume of information on a proteome is not always advantageous because complicates the interpretation of results when compared with a simpler strategy of a more focused project that can generate a lower volume of information. Despite these limitations, the 2-DE is the method most widely used in meat science and can be successfully used to describe muscle proteins.

Many breeds and strains of pigs differ genetically and this is important for improving meat quality. Breeds that were not subject to genetic selection will be reservoirs of alleles that can promote desirable changes in this feature (Serão et al., 2011). The local Brazilian breed Piau is characterized by lower growing with relatively

greater intramuscular fat content. Morphologically, the body composition is distributed harmonically and is considered a genetic bank for not having undergone breeding selection. On the other hand, the commercial breeds among them Landrace, Large White, Pietran and their crosses are characterized by the production of lean meat, low fat deposition and high feed efficiency with excellent productive and reproductive performance. Morphologically, the rear body is more developed and they are responsible for meat production worldwide. Proteomic studies involving local Brazilian breed such as Piau, Canastra, Nilo and Caruncho, are still missing. Moreover, the proteomic profiles of local breeds from other countries have been compared with commercial breeds to better understand the differences between them (Park et al., 2007; Xu et al., 2009; Murgiano et al., 2010). Thus, the aim of the present study was to use proteomics to compare the protein profiles between pigs of different genetic groups using a new method of tissue preservation. Besides, the efficacy of RNAholder® instead liquid nitrogen was evaluated in order to identify proteins. It is believed that this will help to elucidate the biological and functional mechanisms involved in animal muscles and meat quality.

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CHAPTER I

COMPARISON BETWEEN METHODS FOR TISSUE PRESERVATION USING LIQUID NITROGEN AND RNAholder® FOR PROTEOMIC ANALYSIS OF PIG MUSCLE

ABSTRACT

The proteome of porcine skeletal muscle in most cases have been performed based in the 2-dimensional electrophoresis (2-DE) and mass spectrometry analysis. In all studies, the standard method for tissue preservation after slaughtered has been performed using liquid nitrogen. The RNAholder® solution was utilized to compare the efficiency in tissue preservation. Samples from *Longissimus dorsi* muscle from three animals of the same genetic group (Landrace x Large White) at five month of age and average weight 81.9 kg were utilized. Twelve abundant protein spots which were not significantly different between the methods were identified to evaluate the efficacy of the lift in MALDI-TOF/TOF. The results demonstrated the efficacy of the RNAholder® method and the technique of 2-DE was performed. Twenty-five protein spots were significantly different between the two methods. Nine spots were overexpressed in RNAholder® method; three overexpressed when the Liquid Nitrogen was used; 13 protein spots were only present in RNAholder® method. Among them, the glyceraldehyde-3-phosphate dehydrogenase protein and the creatine kinase M-type were identified in RNAholder® group. The results demonstrated that RNAholder® solution presented higher number of proteins probably due to better separation. The solution allows the proteins identification and can be successfully used to preserve protein profiles in porcine muscle in proteomic studies. Besides, the RNAholder® solution presented the advantage of allowing the necessary solubilization of the samples at lower cost and by not requiring freezing muscle.

RESUMO

O proteoma do músculo esquelético de suínos tem sido realizado, na maioria das vezes na eletroforese bidimensional (2-DE) e espectrometria de massas. Em todos os estudos, o método padrão para a preservação do tecido após o abate tem sido a utilização de nitrogênio líquido. A solução de RNAholder® foi utilizada para comparar a eficiência na preservação do tecido. Foram utilizadas amostras de músculos *Longissimus dorsi* provenientes de 3 animais do mesmo grupo genético (Landrace x Large White) com 5 meses de idade e média de peso vivo de 81,9 kg. Doze *spots* de proteínas abundantes que não apresentavam diferença significativa entre os métodos foram selecionados para avaliar a eficiência da ionização no MALDI-TOF/TOF. Os resultados demonstraram a eficácia do método com RNAholder® e a técnica de 2-DE foi realizada. Trinta e três *spots* de proteínas foram significativamente diferentes entre os dois métodos. Doze *spots* foram mais expressos no método do RNAholder®; 4 mais expressos no método com Nitrogênio Líquido; 15 *spots* estavam presentes somente no método com RNAholder® e 2 *spots* estavam presentes somente no método. Entre as proteínas, foram identificados o gliceraldeído-3-fosfato desidrogenase e a creatina kinase Tipo M no grupo do RNAholder®. Os resultados demonstraram que a solução de RNAholder® apresentou maior número de proteínas provavelmente devido à melhor separação. Além disso, esta solução permite a identificação de proteínas e pode ser usada com sucesso para preservar os perfis de proteínas nos estudos proteômicos de músculos de suínos.

INTRODUCTION

Pork production has a great impact on word economy and over the last decade there have been significant improvements of techniques to attend market needs. New researches are made in order to attempt the consumers and to unravel the mechanisms involved in the determination of phenotypic traits. Genome researches consider the genes and the non-coded sequences of DNA and RNA to obtain information about the heritance of organisms. However, this information had shown to be insufficient to related genes to its biological functions. Proteomics have been performed as a tool to unravel molecular and biological mechanisms involved in pork meat quality (Van de Wiel & Zhang, 2007; Laville et al., 2009; Li et al., 2013), meat processing (Luccia et al., 2005; Barbieri & Rivaldi, 2008; Théron et al., 2011), pre-slaughter changes (Morzel et al., 2004; Kwasiborski et al., 2008; Picard et al., 2010) and postmortem changes (te Pas et al., 2009; Bernevic et al., 2011; Nam et al., 2012; Bjarnadóttir et al., 2012). Besides the studies related to the meat, some studies have also focused in proteomic profiles between porcine breeds and crossbreeds (Park, et al., 2007; Xu et al., 2009; Murgiano et al., 2010); proteomic profiles of different tissues (Hwang et al., 2004; Mach et al., 2010); proteomic during myogenesis (Lefaucheur & Ecolan, 2007); proteomic at different ages (Hollung, et al., 2008; Xu et al., 2012); nutrition (Sarr et al., 2010; Théron et al., 2011); proteomic in different animal weigh (Lametsch et al., 2006; Wang et al., 2008;) and effects of genetic background, rearing environment and gender (Kwasiborski et al., 2008b).

Most proteomics tools adopted for the analysis of the porcine skeletal muscle are based on 2-dimensional gel electrophoresis (2-DE) and mass spectrometry (Bendixen, 2005; Righetti, et al., 2005; Sayd et al., 2006; Kwasiborski et al., 2008a; Xu, et al., 2009; Murgiano, et al., 2010(Bendixen, 2005; N. K. Kim et al., 2009; Sayd et al., 2006)). The 2-DE technology allows the quantitative display of large numbers of proteins, differences in protein expression and the comparison between experimental treatments. The analysis of the 2-DE method is based initially on the application of liquid nitrogen for tissue preservation. In this proteomic analysis, the samples must be properly preserved in order to avoid protein degradation. The correct protein extraction and solubilization must be performed to guarantee reliable results after 2-DE technique.

Besides, samples should have a high protein concentration and be free of disturbing factors such salt, ionic detergents, nucleic acids, lipids and others (Jiang, et al., 2004).

Despite the success of tissue preservation protocols based in liquid nitrogen, the study of other sample preparation methods prior to proteomic analysis has been reported (Lenchik et al., 2005; Jiang et al., 2004; Mutter et al., 2004; Abbaraju, et al., 2011). Alternative immersion of fresh tissues in RNAlater® has been reported by Abbaraju et al. (2011) to analysis in fish by one-dimensional (1-D) gels. The use of this solution for proteomic purposes in pig was not reported yet.

In Brazil, a reagent named RNAholder® (Bioagency, São Paulo, SP, Brazil) solution is commonly used to evaluate pattern for gene expression (Serão et al., 2011). This solution for RNA stabilization, avoid the degradation of the single-stranded molecule. It is similar to RNAlater® (Ambion, Applied Biosystems, California, US) and it is also an aqueous tissue storage reagent that stabilize and protect RNA in fresh specimens. This reagent works by rapidly infiltrating cells with a high concentration of ammonium sulfate, causing a mass precipitation of cellular proteins, while the cellular structure remains intact (Lader, 2001). It eliminates the need to immediately process or freeze samples and can be stored indefinitely at – 20 °C or below. In this regard, the aim of the present study was to compare the efficacy of the reagent RNAholder® with the standard liquid nitrogen method in preserving porcine skeletal muscles for proteomics based on 2-DE and mass spectrometry.

MATERIALS AND METHODS

All animal procedures were performed according to protocols approved by Committee for Institutional Use of Animals in Research and Training Activities from Universidade Federal de Viçosa (Viçosa, MG – Brazil).

Animals and sampling

The sampled pig population was composed of three female pigs from the Pig Breeding Farm of the Universidade Federal de Viçosa (Viçosa, MG – Brazil). These animals consisted of Commercial Line (Landrace x Large White), and were slaughtered

by electrical stunning at five months of age and average weight 81.9 kg. For each animal, the *Longissimus dorsi* muscle was collected and the tissue preservation was made simultaneously by two different methods in the first 15 min after slaughter. In the first method, the collected muscle samples were immediately frozen in liquid nitrogen and kept at -80°C until subsequent analysis. In the second method, small pieces of muscle around (0.5 x 0.5 cm) were immersed in 50 mL tubes at weight:volume of 1:5 of RNAholder® (Bioagency, São Paulo, SP, Brazil) stored at 4°C for 24hs and then kept at -20°C until the protein extraction.

Extraction of proteins for the Liquid Nitrogen preservation method

Protein samples were prepared from porcine muscle tissues according to Xu, et al. (2009) method. About 100 mg of the frozen *Longissimus dorsi* was placed in a mortar, added of liquid nitrogen and grounded thoroughly to a very fine powder with a pestle. The obtained powder was macerated with 1 mL of sample preparation buffer {7 *M* urea, 2 *M* thiourea, 4% (w/v) 3-3 [(cholamidopropyl)dimethilammonio]-1-propanesulfonate (CHAPS); 1% (w/v) dithiothreitol (DTT); 2% (v/v) immobilized pH gradient (IPG) buffer, pH 3 to 10; 10 µL benzamidine hydrochloride hydrate, and 10 µL phenylmethanesulfonyl fluoride (PMSF)}. The mixture was transferred to 2 mL-sterile tubes and ultrasonicated for 6 times (15 s each time) and then incubated by 60 min at room temperature vortexing each 20 min throughout this period. The tubes were centrifuged at 20,000 x *g* for 45 min at 4°C and the supernatant was collected and stored at -80°C until analysis.

Extraction of proteins for the RNAholder® preservation method

The muscle samples were weighed (100 mg) after gently removing as much as possible the RNAholder® solution by rubbing the tissue over an aluminum foil. In order to improve cleaning of the tissue sample prior to protein extraction procedure, washing tests were performed as the following scheme:

Table 1. Tests performed in *Longissimus dorsi* muscle

Time of washing[1]	Number of washes[2]	
	1	3
No washed	0	0
Liquid Nitrogen	0	0
5 s	5''x1	5''x3
1 min	1'x1	1'x3
5 min	5'x1	5'x3
10 min	10'x1	10'x3

[1]Time that samples were soaked in PBS solution per wash
[2]Number of subsequently washes

New solutions of PBS were used when the tissues were washed more than once, except for the control group that was no washed. After washing, the muscles were homogenized with liquid nitrogen as previously described. The tissue powder was homogenized with 1 mL of sample preparation buffer {7 *M* urea, 2 *M* thiourea, 4% (w/v) 3-3 [(cholamidopropyl)dimethilammonio]-1-propanesulfonate (CHAPS); 1% (w/v) dithiothreitol (DTT); 2% (v/v) immobilized pH gradient (IPG) buffer, pH 3 to 10; 10 µL benzamidine hydrochloride hydrate and 10 µL phenylmethanesulfonyl fluoride (PMSF)}. The mixture was transferred to 2 mL-sterile tubes and ultrasonicated for 6 times (15 s each time) and then incubated by 60 min at room temperature vortexing each 20 min throughout this period. The tubes were centrifuged at 20,000 x *g* for 45 min at 4°C and the supernatant was collected and stored at -80°C until analysis.

Protein quantitation

The protein concentration of all assayed sample was determined using the PlusOne 2-D Quant Kit (GE Healthcare Bio-Sciences, Piscataway, USA). BSA was used as the standard protein for a calibration curve. The treatments were analyzed according to Factorial Experiment with four times (5 s, 1 min, 5 min, and 10 min), and two washes (once and three times). The Tukey's test was performed to evaluate differences between means. Statistical analysis was performed using SAS 9.0 for Windows (Statistical Analysis System Institute, Inc. Cary, USA). Based on the protein

concentration, the control group, or samples from RNAholder® that were not washed with PBS solution, was chosen to continue the study of proteomics.

Mono-dimensional gel electrophoresis

To evaluate de efficacy of quantification, mono-dimensional SDS-PAGE (Sodium Dodecyl Sulfate Polyacrilamide Gel Eletrophoresis) in 7 cm wide and 12% gels were developed. The amount of 30 µg protein per treatment was loaded per lane. The total time running was about 2 h. After running, gels were stained according to Coomassie Blue R-250 procedure for 2 h. The gels were then immersed in distaining solution with 25% methanol, 7.5% acetic acid, and double-distilled water for 24 h. The stained gels were transferred to 5% acetic acid solution until image analysis.

First dimension of the 2-DE

For the separation of proteins in the first dimension (isoelectric focusing - IEF) of the 2-DE, samples from both methods of tissue preservation were solubilized in rehydration solution [DeStreak Rehydration Solution (GE Healthcare Bio-Sciences), 1% (w/v) DTT, and 2% (v/v) immobilized pH gradient (IPG) buffer pH 3 to 10]. For the preparative gels, aliquots of 1,000 µg of proteins were loaded to the mixture and centrifuged at 12,000 x *g* for 1 min at 20°C and the supernatants were used for rehydration in Immobilized pH Gradient (IPG) strips (GE Healthcare Bio-Sciences). The IPG strips of 24 cm, pH 3-10, were rehydrated in 450 µL of the protein solution for 10 h at room temperature in the Immobiline DryStrip Reswelling Trays (GE Healthcare Bio-Sciences). The IEF was performed using Ettan IPGphor III System (GE Healthcare Bio-Sciences) apparatus at 20°C.

The running conditions using Ettan IPGphor III System were different for samples between both methods of tissue preservation. In the standard method (liquid nitrogen), running conditions was similar to the GE protocol (GE Healthcare Bio-Sciences), except for the first step. The current limit was 75 µA per strip and 300 V was applied in the initial step during 12 h followed by step and hold until 500 V (12 h); gradient mode at 1,000 V (1 h) followed by another gradient until 10,000 V (3 h) and

step and hold mode at 10,000 V (2:45 h). The total running time for this program was 19:45 h and reached a total of 43,795 kVh. After IEF was completed, the Immobiline DryStrip gels were stored in Equilibration tubes (GE Healthcare Bio-Sciences) at -80°C until proceed to the second dimension.

Samples from RNAholder® treatment, the running conditions using Ettan IPGphor III System were similar to the GE protocol (GE Healthcare Bio-Sciences), except for the first and the last step. The current limit was 75 µA per strip and 300 V was applied in the initial step during 12 h followed by step and hold until 500 V (1 h); gradient mode at 1000 V (1 h); another gradient mode until 10000 V (3 h) and step and hold mode at 10000 V (6:45 h). The total running time for this program was 23:45 h and reached a total of 59939 kVh. After IEF, the Immobiline DryStrip gels was stored in Equilibration tubes at -80°C until proceed to the second dimension.

Second dimension of the 2-DE

Focused IPG strips were equilibrated by 15 min in SDS equilibration buffer solution (6 *M* urea, 75 m*M* Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue and 1% DTT, and then for an additional 15 min in the same equilibration buffer and 4% iodoacetamide instead of DTT. After equilibration, proteins were separated in the second dimension with the Etan DALTsix (GE Healthcare Bio-Sciences) apparatus on 12.5% SDS-PAGE gels at 8°C. The running for samples obtained from the two preservation methods were performed separately from each other, despite the same conditions described as follow. Electrophoresis was performed at constant power, in two steps. In the first step, 10 mA per gel was applied for 1 h, and in the second step, 40 mA per gel. After electrophoresis development, the gels were immersed in solution with 40% ethanol, 10% acetic acid, and 50% double-distilled water for 24 h and then stained according to Colloidal Coomasie Blue G-250 procedure by 48 h. The stained gels were transferred to 5% acetic acid solution that was exchanged twice more at each 24 h until subsequent image analysis.

Image and data analysis

The 2-DE gels were scanned on an Image Scanner using Lab Scan program (GE Healthcare Bio-Sciences) at 300 dots per inch. Spot detection and quantification were performed with Image Master 2D Platinum software version 7.0 (GE Healthcare Bio-Sciences). The parameters for spot detection were smooth factor 2.0, saliency 50.0 and minimal area 50 pixels. The reference gel was used considering the greater number of the spots and then used for matching of corresponding protein spots between gels. For comparative image analysis, the images were grouped after the intensity of the individual spots has been analyzed and compared within and between the images groups of treatments ("Liquid Nitrogen" *versus* "RNAholder®"). The changes in spot patterns revealed by computer-based image analysis were individually inspected and confirmed. The Student's *t*-test was used for statistical analysis with p value ≤ 0.05 and 1.5 match count.

In order to evaluate the efficacy of the RNAholder® method related to ionization, activation and detection, and consequently to its capacity for protein identification in the MALDI TOF/TOF analysis, 12 protein spots were selected to be analyzed from the gels of both methods.

In-gel digestion of proteins

The protein spots of interest were cut out from preparative gels using pipet tips of 1,000µL and extracted from gels by gently suction. The gels pieces were dehydrated with 50% acetonitrile (v/v) and 25 mM ammonium bicarbonate, pH 8.0, and dried at room temperature. The trypsinization were performed using a modified method based on Shevchenko, et al., (2006). The gel pieces were alkylated with 200 mM iodoacetamide in 100 mM ammonium bicarbonate pH 8.0 for 30 min at room temperature and then digested with 25 $\mu g.\mu L^{-1}$ of trypsin solution (Tripsin Porcine, Mass Spectrometry Grade, Promega). In-gel tryptic degradation was performed overnight at 37 °C. The samples were desalting using C18 micro columns (Zip Tip, Millipore, Ireland) to avoid interference in crystallization of the samples with the matrix used in for MALDI-TOF/MS analysis, and were then stored at -20 ^{o}C until subsequent analysis.

Protein identification

The protein identification was performed using matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/MS). The sample solution was applied to the matrix α-cyano-4-hydroxycinnamic acid solution (5 $\mu g.mL^{-1}$ in 50% acetonitrile and 0.1% TFA) onto the MALDI-TOF/MS target (steel plate MTP Anchor Chip TM 600/384 TF, Bruker Daltonics). The MALDI-TOF spectra were calibrated using trypsin Promega porcine peptide signals and matrix ion signals by Bruker Daltonics flexAnalysis Software; MALDI analysis was performed by a feedback control systems (Ultraflex III MALDI TOF/TOF system, Bruker, Germany). The MALDI-TOF/MS analyses were performed with Reflective Positive Peptide method. Peptide masses were searched against the NCBI and SwissProt database with 0.5 error using the Mascot program (http://www.matrixscience.com) and MASCOT Peptide Mass Fingerprinting database search. The initial search variables for MS analysis allowed a single trypsin missed cleavage, no restriction on protein mass, carbamidomethyl (C) for fixed modifications, oxidation (M) for variable modifications, peptide mass tolerance of 0.5 and the taxonomic search space was restricted to mammalian. The protein identification by MS/MS analyses (MALDI-TOF/TOF) was performed using LIFT method. The peptide ions were searched against the NCBI and SwissProt database with 0.5 error using the Mascot program (http://www.matrixscience.com) and MASCOT MS/MS Ions Search database. The search variables for MS/MS analysis allowed a single trypsin missed cleavage; peptide charge 1+; carbamidomethyl (C) for fixed modifications; oxidation (M) for variable modifications; 0.5 peptide mass tolerance; 0.5 MS/MS tolerance and the taxonomic search space was restricted to mammalian. Identities with probability-based ($p<0.05$) in SwissProt database and scores > 46 were significant to NCBInr.

RESULTS AND DISCUSSION

Protein concentration of all treatments is presented in Table 2. The use of PBS apparently did not affect the protein concentration, so the control group (*RNAholder®* group without washing) was chosen to proceed the 2-DE gels.

Table 2. Protein concentration of all tested groups

Treatment	Protein concentration (mg/mL)*
5''x1	21,02 ± 5,77 a
5''x3	19,62 ± 2,86 a
1'x1	20,86 ± 5,41 a
1'x3	16,42 ± 2,94 a
5'x1	19,68 ± 2,95 a
5'x3	16,11 ± 3,31 a
10'x1	20,99 ± 3,23 a
10'x3	13,49 ± 2,01 a
No washed	22,42 ± 4,25
Liquid Nitrogen	26,27 ± 2,78

* Treatments followed by the same letter indicate no significant difference at $p<0.05$ by Tukey's test.

The use of RNAholder® prolonged the time for the IEF (Figure 2), similarly to protein samples enriched in salt. Samples from RNAholder® treatment presented greater resistance to run, evidenced by a black line on the strip (red arrow in Figure 1). The running conditions took into account the displacement of this mark until the end of the strips. Therefore the focusing time was the same that was used in standard method for all steps of Voltage modes, excepted in the final step programming that was longer than GE protocol (GE Healthcare Bio-Sciences). The final step Voltage mode was 10,000 V for 6:45 h, in a total running time of 23:45 h reaching 59,939 kVh. Salt in the IPG strip results in high strip conductivity and the process of focusing proteins will be delayed until the ions have been moved to the ends of the strips (Görg, 2004).

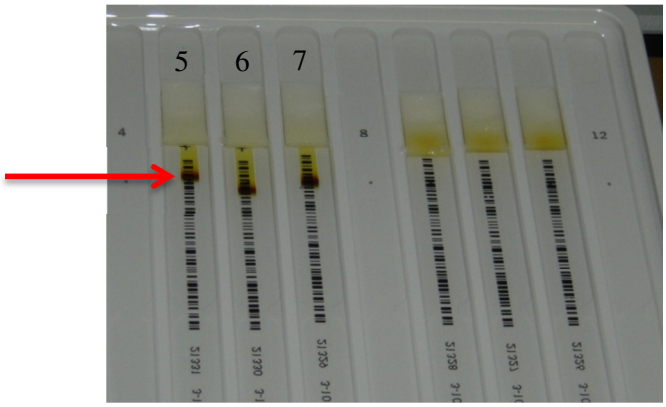

Figure 2. Immobiline DryStrip gels from samples obtained from RNAholder® procedure. After running, the black line on strips was noted in the same sample (triplicates, lanes 5 to 7, indicated by the red arrow).

The 12 protein spots selected to compare the capacity of identification are presented in Figure 3. The abundant protein spots selected were not significantly different between methods.

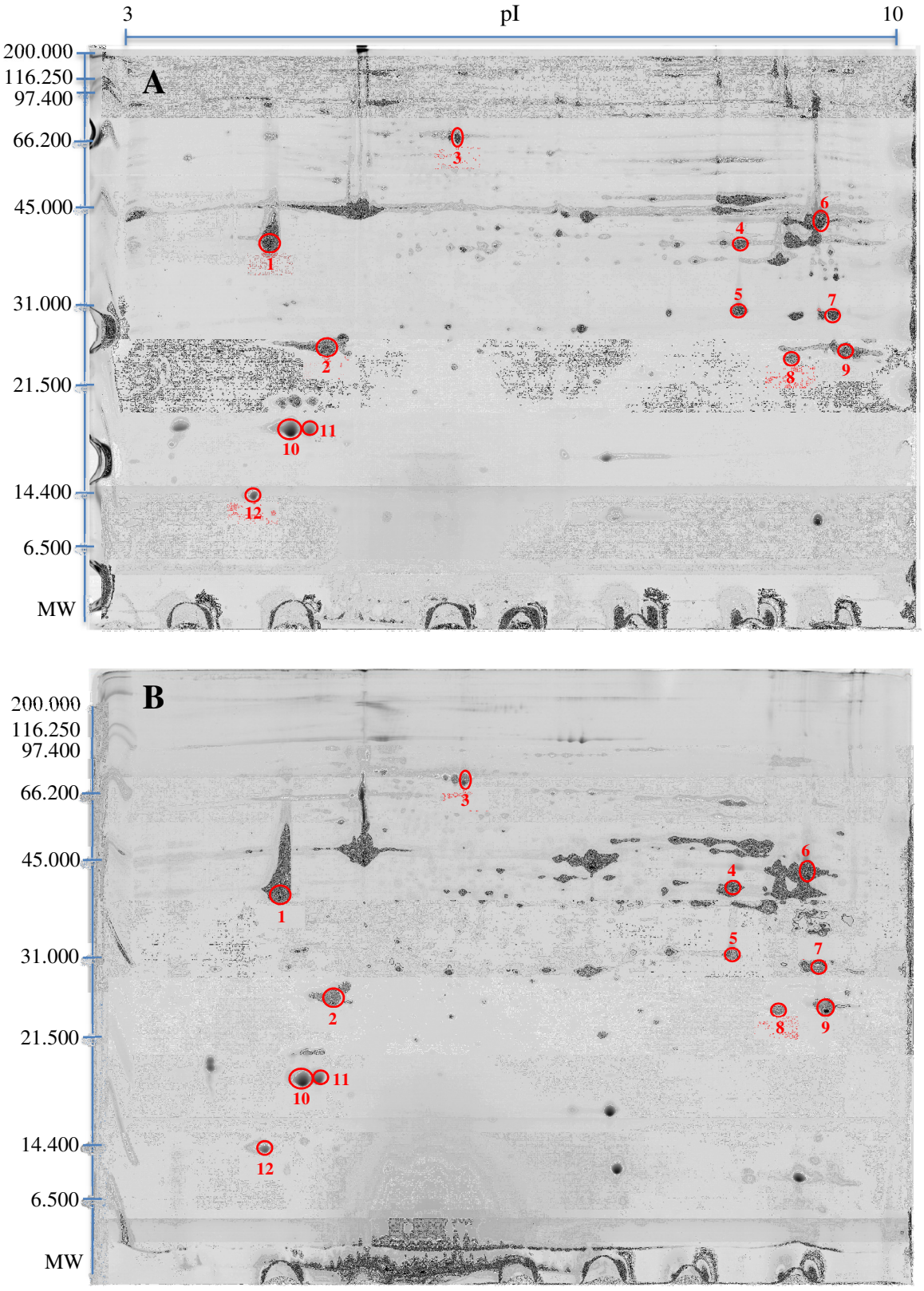

Figure 3. Scanned 2-DE image of *Longissimus dorsi* muscle separated using an IPG pH 3-10 in the first dimension (24cm, GE Healthcare Bio-Sciences, Uppsala, Sweden) and 12.5% SDS gel in the second dimension. Circles show 12 selected proteins extracted from gels. The same spots were extracted from (A) *Liquid Nitrogen* method and (B) *RNAholder*® method.

The identification and information related to the validity of search results are shown in Table 3. All the 12 pairs of spots from the two groups of tissue preservation were successfully identified by MALDI-TOF/TOF. Among them, nine spots were identified by matching peptide data to porcine protein sequences in the database, whereas three spots were identified due to interspecies homology to *Mus musculus, Bos Taurus* and *Oryctolagus cuniculus*. The protein name, accession number, Mascot score, matched peptides, % sequence coverage, source and theoretical pI and MW were derived from database.

Table 3. Proteins identified in gels from both methods for tissue preservation

Spot no.	Identified proteins[1]	Experimental pI/MW[2]	Theoretical pI/MW[3]	Matched peptides/% sequence coverage[4]	Mascot score	accession no.[5]	Source[5]	Database
1	Tropomyosin 2 beta	4.02/40402	4.65/33031	17/40	86	gil50190	Mus musculus	NCBI
2	Myosin light chain 1f	4.65/28308	4.90/21019	3/35	166	gil117660874	Sus scrofa	NCBI
3	Albumin	5.92/72272	5.92/71362	30/49	110	gil833798	Sus scrofa	NCBI
4	Troponin T, fast skeletal muscle type	8.80/40402	5.99/32107	10/29	88	TNNT3_BOVIN	Bos taurus	SwissProt
5	Carbonic anhydrase 3	8.80/32215	7.72/29678	11/56	88	gil56711366	Sus scrofa	NCBI
6	Aldolase C	9.60/43339	9.06/38993	6/41	119	gil229506	Oryctolagus cuniculus	NCBI
7	Phosphoglycerate mutase 2	9.80/31391	8.86/28830	16/50	139	gil201066358	Sus scrofa	NCBI
8	Adenylate kinase isoenzyme	9.38/27448	8.38/21739	12/67	93	KAD1_PIG	Sus scrofa	SwissProt
9	Troponin I, fast skeletal muscle	9.96/28026	9.02/21491	8/26	96	gil73853890	Sus scrofa	NCBI
10	Myosin regulatory light chain 2, skeletal muscle isoform	4.31/21572	4.82/19066	15/70	131	gil117660856	Sus scrofa	NCBI
11	Myosin regulatory light chain 2, skeletal muscle isoform	4.50/22008	4.82/19066	13/69	134	gil117660856	Sus scrofa	NCBI
12	myosin light chain	3.88/15776	4.63/16833	5/39	58	gil5834684	Sus scrofa	NCBI

[1]Protein name
[2]MW = molecular weight; p*I* = isoelectric point
[3] Theoretical p*I* and MW derived from database in relation to identified proteins
[4]The number of matched peptides in the database search and the minimum coverage of the matched peptides in relation to the full-length sequence
[5]The accession number and source from the database search

Then, the results demonstrated the efficacy of the RNAholder® method for protein extraction, separation, and identification. The proteomic analysis was performed in order to compare the protein profiles between methods. Proteins from the *Longissimus dorsi* muscle from the Commercial line pigs, after separation in 2-DE gels, evidenced a total of 1037 spots that were considered for the statistical analysis. According to differences between methods of tissue preservation, a total of 234 protein spots were significantly changed as validated by ANOVA and *P*-values < 0.05. After verification of individual spots, 25 protein spots were significantly different between both methods of tissue preservation and were selected for protein identification. Among the 25 selected protein spots, nine spots were overexpressed in *RNAholder®* group; three spots were overexpressed in *Liquid Nitrogen* group, 13 spots were present only in *RNAholder®* method and two spots were present only in *Liquid Nitrogen* method. Of these, 16 proteins could not be identified due to lack of sequence similarities in the database and six proteins could not be extracted from the preparative gels due to low abundance or poor separation. The representative gels and the identified proteins are presented in Figure 3.

The metabolic enzymes glyceraldehyde-3-phosphate dehydrogenase protein and two isoforms of creatine kinase M-type were identified in *RNAholder®* method. The identification and the search results are shown in Table 4. The glyceraldehyde-3-phosphate dehydrogenase protein presented only in *RNAholder®* method and the creatine kinase were overexpressed in relation to *Liquid Nitrogen* method.

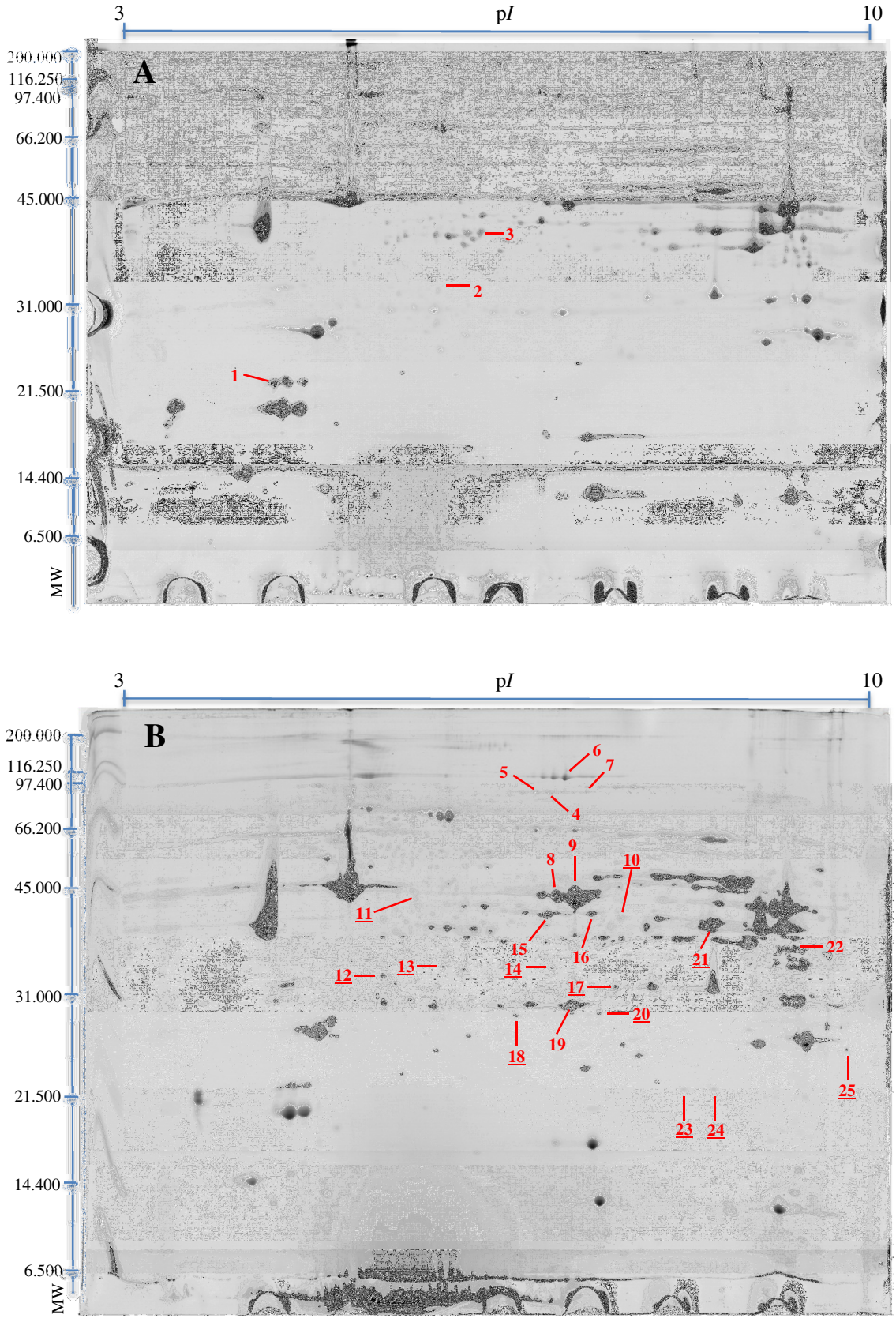

Figure 3. Representative 2-DE images of *Longissimus dorsi* muscle sample from (A) Liquid Nitrogen and (B) RNAholder® method of tissue preservation. Proteins are separated by pH 3-10 in the first dimension and 12.5% SDS-PAGE in the second dimension. The overexpressed proteins are marked in red with respective numbers within groups. The proteins present only in RNAholder method are marked in red and subscribed.

Table 4. Differentially expressed muscle proteins identified by 2-Dimensional gel electrophoresis and MALDI-TOF/TOF mass spectrometry[1]

Spot no.	Identified proteins[2]	Experimental p*I*/MW[3]	Theoretical p*I*/MW[2]	Matched peptides/% sequence coverage[4]	Mascot score	Accession[5]	Source[2]	Database	Ratio RNAh/ N_2[6]	*P*-value[7]
Metabolic enzymes										
8	Creatine kinase M-type	7.19/44514	6.44/43205	25/53	104	KCRM_PIG	Sus scrofa	SwissProt	2.65	0.0006
9	Creatine kinase M-type	7.01/45065	6.61/43260	13/28	83	KCRM_PIG	Sus scrofa	SwissProt	3.93	0.0005
22	Glyceraldehyde-3-phosphate dehydrogenase	8.64/39285	8.50/36073	12/14	52	G3P_BOVIN	Bos Taurus	SwissProt	RNAh	0.0007

[1]Abbreviations: RNAh = RNAholder® group; N_2 = Liquid Nitrogen group
[2]Protein name, theoretical p*I* and MW and accession numbers were derived from database.
[3]MW = molecular weight; p*I* = isoelectric point
[4]The number of matched peptides in the database search and the minimum coverage of the matched peptides in relation to the full-length sequence
[5]The database search
[6]Relative differences in 2-dimensional gels electrophoresis gel spot intensities: RNAh spot intensity/N_2 spot intensity. RNAh = spots presents only in RNAholder® group.
[7]*P*-value from the Student's test.

The glyceraldehyde-3-phosphate dehydrogenase protein (spot 22 in Figure 3B) is a metabolic enzyme involved in glycolytic pathway. It has been reported that this enzyme also envolved in glycolytic pathway in *Adductor* muscle (Kristin Hollung et al., 2008); *Semimembranosus* muscle (Sayd et al., 2006) and *Longissimus dorsi* muscle (R Lametsch et al., 2006) . Although this enzyme has been identified only in *RNAholder®* method of tissue preservation, the glyceraldehyde-3-phosphate dehydrogenase is an endogenous protein known to be present in skeletal muscle. It is possible that this solution can be more efficient to separate the molecules in two-dimensional electrophoresis as shown in the image gels represented in Figure 2. The better separation of the protein spots can also be observed due to overexpression of the two isoforms of the creatine kinase (spots 8 and 9 of Figure 3B). Since the samples were from the same muscle and the same animal and the patterns for the separation of proteins are different for the two methods, it is possible to conclude that the *RNAholder®* method can separate more efficiently the proteins. The higher number of protein spots statistically detected corroborates this hypothesis.

The gels containing *RNAholder®* samples presented more proteins that the frozen method of tissue storage, probably due to better precipitation of cellular proteins.

The proteins present different solubility in different salt types and concentration. Besides, some extrinsic proteins are anchored to cellular structures by electrostatic interactions that can be released in the presence of salt.

The choice of the extraction method can determine which proteins are extracted and which will not be considered (Hollung et al., 2007). The 2-DE analysis has some limitations and disadvantages. Proteins with high (> 150 kDa) or low (<10 kDa) molecular weight as well as proteins with isoelectric points of extreme values, particularly basic proteins, are normally not detected by 2-DE gels. Another drawback is that hydrophobic proteins such as membrane proteins and proteins with a high molecular weight, which due to difficult solubilization during extraction and due to precipitation during electrophoresis, do not appear in the gel. For this reason, the technique of 2-DE gels hinders investigations of many muscle structural proteins such as myosin heavy chain, titin and nebulin, since they have a molecular weight above 150 kDa (Lametsch, 2011).

The proteins profile could be complemented using another technique. According to Lippolis & Reinhardt (2008), the range of protein expression complicates detection of low abundance proteins in typical biological samples. In this case, the shotgun approach could be able to analyze a large complex proteomes for changes in protein presence, expression, or modification. Shotgun proteomics analysis usually involves differential isotope labeling of proteins. The labeled peptides are separated by multidimensional liquid chromatography (LC), and resolved peptides are analyzed using MS. Besides, the shotgun proteomic methods could resolve some limitations faced in traditional gel-based proteomic approaches, as the inability to analyze highly basic/hydrophobic proteins or the detection of proteins that lie on the extreme ends of MW and pI (Aggarwal, et al., 2006). Another alternative for a more complete identification of proteins is the fractionation of the proteomes into less complex mixtures before mass spectrometry. There are several fractionation schemes that can separate proteins or peptides by their various physical attributes subsequent to or in place of subcellular fractionation. These schemes may include enrichment of proteins using molecules with specific binding affinities to isolate away a group of proteins, gel electrophoresis, or various chromatographies. Besides, the fractionation strategies can be used individually or in combination to improve detection of small abundance proteins (Lippolis & Reinhardt, 2008).

CONCLUSIONS

The proteomic analysis based on 2-DE may be developed by using the RNAholder® solution without washing with PBS solution to preserve muscle samples. This reagent presents advantages as: immediately immersion of the samples in solution; unnecessary use of liquid nitrogen for snap-freezing samples or the use of ultra-freezer to storage samples at -80°C; allows long periods of sample storing and, in special, it is compatible using in another biological analysis to functional genomics when compared with the *Liquid Nitrogen* method evaluated. It may be pointed that the gels presented more protein spots when compared with the *Liquid Nitrogen* standard procedure. Moreover, proteins were equally identified by MALDI-TOF/TOF. This procedure enables to perform researches in material previously conserved in this solution for transcriptomic or other analysis.

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CHAPTER II

COMPARISON OF PIG MUSCLE PROTEOME PROFILES BETWEEN LOCAL BRAZILIAN BREED PIAU AND COMMERCIAL LINE

ABSTRACT

The local Brazilian breed Piau and the Commercial line (Landrace x Large White) present differences in muscle growth, intramuscular fat content and meat quality. The use of RNAholder® solution to preserve sample tissue was performed. The comparison between proteomic profiles of these two breeds were realized at 90 kg of body weight. Proteins from the *Longissimus dorsi* muscle from Commercial line and Piau breed were separated by 2-dimensional electrophoresis (2-DE) technology and mass spectrometry. At the protein level, 35 protein spots were significantly different between the two genetic groups. Six spots were overexpressed in Piau breed; two overexpressed in Commercial line; three protein spots were present only in Piau breed and 24 spots were present only in Commercial line. The protein abundance indicated differences between genetic groups in structural proteins; cell-defense and stress-related proteins. The results suggested that the local breed Piau have oxidative metabolism instead the glycolytic one, observed in Commercial line. The use of RNAholder® allowed the protein identification, but new investigations in proteomics profiles could provide more information in these genetic groups.

RESUMO

A raça naturalizada brasileira Piau e linha Comercial (Landrace x Large White) apresentam diferenças no crescimento muscular, gordura intramuscular e qualidade de carne. A solução de RNAholder® foi utilizada para preservar amostra de tecido nestes dois grupos genéticos. A comparação entre os perfis proteômicos destes dois grupos genéticos foi realizada aos 90 kg de peso vivo. As proteínas do músculo *Longissimus dorsi* da linha Comercial e Piau foram separadas pela tecnologia de eletroforese bidimensional (2-DE) e espectrometria de massas. Das proteínas, 35 *spots* foram significativamente diferentes entre os dois grupos genéticos. Seis *spots* foram mais expressos na raça Piau; dois mais expressos na raça Comercial; três *spots* de proteínas estavam presentes somente na raça Piau e 24 somente na linha Comercial. A abundância de proteínas indicou diferença entre os grupos genéticos divergentes nas proteínas estruturais; defesa celular e proteínas relacionadas ao estresse. Os resultados sugerem que a raça local Piau tem um metabolismo oxidativo ao invés de glicolítico, observado nas raças Comerciais. O uso do RNAholder® permitiu a identificação da proteína, porém novas investigações de perfis proteômicos podem fornecer mais informações nestes grupos genéticos.

INTRODUCTION

One of the goals in livestock is the production of food for the human consumption, among them meat that is responsible for a large amount of protein supply. Pork is one of the most consumed worldwide and it is related to quality meat. The intramuscular fat (IMF) content or marbling is positively associated to juiciness and tenderness of meat. Pig breeds that have been intensively selected growth traits, have higher meat content and are widely used for commercial production throughout the world. Although this improvements, the intensive selection led to deterioration of meat quality (Xu et al., 2009; Zhao, et al., 2010; Li et al., 2013) been the blandness and the low-fat of meat the most undesirable traits for consumers. Breeds that have never undergone genetic selection may be suitable reservoirs of genes that can promote desirable changes in these traits. The local Brazilian pig breed Piau has slower growth rate and higher IMF content (Serão et al., 2011). In this way, comparison of skeletal muscle between these 2 divergent genetic groups might help understand the molecular mechanisms underlying differences in meat quality.

In meat science, proteomics can be considered a relatively new tool and has been used in several studies to unravel molecular and biological mechanisms involved in pork meat quality (Van de Wiel & Zhang, 2007; Laville et al., 2009; Li et al., 2013), meat processing (Luccia et al., 2005; Barbieri & Rivaldi, 2008; Théron et al., 2011), pre-slaughter changes (Morzel et al., 2004; Kwasiborski et al., 2008; Picard et al., 2010) and postmortem changes (te Pas et al., 2009; Bernevic et al., 2011; Nam et al., 2012; Bjarnadóttir et al., 2012). Besides the studies related to the meat, some studies have also focused in proteomic profiles between porcine breeds and crossbreeds (Park, et al., 2007; Xu et al., 2009; Murgiano et al., 2010); proteomic profiles of different tissues (Hwang et al., 2004; Mach et al., 2010); proteomic during myogenesis (Lefaucheur & Ecolan, 2007); proteomic at different ages (Hollung, et al., 2008; Xu et al., 2012); nutrition (Sarr et al., 2010; Théron et al., 2011); proteomic in different animal weights (Lametsch et al., 2006; Wang et al., 2008;) and effects of genetic background, rearing environment and gender (Kwasiborski et al., 2008b).

Appropriate sample preparation is essential for obtained reliable results in a proteomic analysis. It is advisable to keep sample preparation as simple as possible to avoid protein losses (Hollung, et al., 2007). Rapidly freezing tissue samples in liquid

nitrogen is the standard method of tissue preservation avoiding protein modifications and degradations. Despite the success of this method, the study of other sampling methods prior to proteomic analysis has been reported using RNAlater® (Ambion, Applied Biosystems, California, US) as an alternative to reduce problems concerns of handling liquid nitrogen during sampling in other organisms than pig (Jiang et al., 2004; Mutter et al., 2004; Lenchik, et al., 2005; Abbaraju, et al., 2011). In Brazil, a product named RNAholder® (Bioagency, São Paulo, SP, Brazil) solution is commonly used to evaluate pattern for expression genes (Serão et al., 2011). This stabilization solution is similar to RNAlater® and it is aqueous tissue storage reagents that stabilize and protect RNA in fresh specimens. This reagent functions by rapidly infiltrating cells with a high concentration of ammonium sulfate, causing a mass precipitation of cellular proteins and remaining intact the cellular structure (Lader, 2001). The comparison between liquid nitrogen and RNAholder® method of tissue preservation in order to perform proteomic based in 2-DE electrophoresis and mass spectrometry was performed previously. In this study, the use of RNAholder® to preserve tissues samples was performed in order to compare the proteomic profiles between two genetically divergent genetic groups.

MATERIALS AND METHODS

All animal procedures were performed according to protocols approved by Committee for Institutional Use of Animals in Research and Training Activities from Universidade Federal de Viçosa (Viçosa, MG – Brazil).

Animals and sampling

The sampled pig population was composed of six females pigs from the Pig Breeding Farm of Universidade Federal de Viçosa (Viçosa, MG – Brazil). Of these, three animals consisted of Commercial Line (Landrace x Large White) and three local Brazilian breed Piau. They were slaughtered by electrical stunning at 90 kg of body weight and average of 186 days of age for Piau breed and 141 days old for Commercial line. For each of these animals the *Longissimus dorsi* muscle was collected and the tissue preservation was made within 15 min after slaughter. The small pieces of muscle

(around 0.5 x 0.5 cm) were immersed in 50 mL tubes at weight:volume of 1:5 of RNAholder® (Bioagency, São Paulo, SP, Brazil) stored at 4°C for 24hs and then kept at -20°C until extraction of proteins.

Extraction of proteins

The muscle samples were weighed after gently removed as much as possible the RNAholder® solution rubbing the tissue over an aluminum foil. The amount of 100 mg of *Longissimus dorsi* muscle was frozen with liquid nitrogen and powered with mortar and pestle until grounded to fine powder. The tissue powder was homogenized with 1 mL of sample preparation buffer {7 *M* urea, 2 *M* thiourea, 4% (w/v) 3-3 [(cholamidopropyl)dimethilammonio]-1-propanesulfonate (CHAPS); 1% (w/v) dithiothreitol (DTT); 2% (v/v) immobilized pH gradient (IPG) buffer, pH 3 to 10; 10 µL benzamidine hydrochloride hydrate and 10 µL phenylmethanesulfonyl fluoride (PMSF)}. The mixture was transferred to 2 mL sterile tubes and ultrasonicated 6 times (15 s per time) and then incubated for 60 min at room temperature vortexing every 20 min during this period. The tubes were centrifuged at 20,000 x *g* for 45 min at 4°C. The supernatant was collected and stored at -80°C until analysis. The protein concentration was determined using the PlusOne 2-D Quant Kit (GE Healthcare Bio-Sciences, Piscataway, USA). BSA was used as a standard curve. Protein concentration was 11.10 ± 1.15 mg/mL for Commercial line group and 15.51 ± 1.86 mg/mL for Piau breed.

First dimension of the 2-DE

For the separation of proteins in the first dimension (isoelectric focusing - IEF), the samples were solubilized in rehydration solution [DeStreak Rehydration Solution (GE Healthcare Bio-Sciences), 1% (w/v) DTT and 2% (v/v) immobilized pH gradient (IPG) buffer pH 3 to 10]. For the preparative gels, aliquots of 1,000 µg of proteins were loaded to the mixture and centrifuged at 12,000 x *g* for 1 min at 20°C and the supernatants were used for rehydration in Immobilized pH Gradient (IPG) strips (GE Healthcare Bio-Sciences). The IPG strips of 24 cm, pH 3-10, were rehydrated in 450 µL of the protein solution for 10 h at room temperature in the Immobiline DryStrip

Reswelling Trays (GE Healthcare Bio-Sciences). The IEF was performed using Ettan IPGphor III System (GE Healthcare Bio-Sciences) apparatus at 20°C.

The running conditions using Ettan IPGphor III System were similar to the GE protocol (GE Healthcare Bio-Sciences), except for the first and the last step. The current limit was 75 µA per strip and 300 V was applied in the initial step during 12 h followed by step and hold until 500 V (12 h); gradient mode at 1000 V (1 h); another gradient mode until 10000 V (3 h) and step and hold mode at 10000 V (6:45 h). The total running time for this program was 23:45 h and reached a total of 59939 kVh. After IEF, the Immobiline DryStrip gels was stored in Equilibration tubes at -80°C until proceed to the second dimension.

Second dimension of the 2-DE

Focused IPG strips were equilibrated by 15 min in SDS equilibration buffer solution (6 *M* urea, 75 m*M* Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue and 1% DTT, and then for an additional 15 min in the same equilibration buffer and 4% iodoacetamide instead of DTT. After equilibration, proteins were separated in the second dimension with the Etan DALTsix (GE Healthcare Bio-Sciences) apparatus on 12.5% SDS-PAGE gels at 8°C. The running for samples obtained from the two preservation methods were performed separately from each other, despite the same conditions described as follow. Electrophoresis was performed at constant power, in two steps. In the first step, 10 mA per gel was applied for 1 h, and in the second step, 40 mA per gel. After electrophoresis development, the gels were immersed in solution with 40% ethanol, 10% acetic acid, and 50% double-distilled water for 24 h and then stained according to Colloidal Coomasie Blue G-250 procedure by 48 h. The stained gels were transferred to 5% acetic acid solution that was exchanged twice more at each 24 h until subsequent image analysis.

Image and data analysis

The 2-DE gels were scanned on an Image Scanner using Lab Scan program (GE Healthcare Bio-Sciences) at 300 dots per inch. Spot detection and quantification were

performed with Image Master 2D Platinum software version 7.0 (GE Healthcare Bio-Sciences). The parameters for spot detection were smooth factor 2.0, saliency 50.0 and minimal area 50 pixels. The reference gel was used considering the greater number of the spots and then used for matching of corresponding protein spots between gels. For comparative image analysis, the images were grouped after the intensity of the individual spots has been analyzed and compared within and between the images groups of treatments ("Liquid Nitrogen" *versus* "RNAholder®"). The changes in spot patterns revealed by computer-based image analysis were individually inspected and confirmed. The Student's *t*-test was used for statistical analysis with p value ≤ 0.05 and 1.5 match count.

In-gel digestion of proteins

The protein spots of interest were cut out from preparative gels using pipet tips of 1,000µL and extracted from gels by gently suction. The gels pieces were dehydrated with 50% acetonitrile (v/v) and 25 mM ammonium bicarbonate, pH 8.0, and dried at room temperature. The trypsinization were performed using a modified method based on Shevchenko, et al., (2006). The gel pieces were alkylated with 200 mM iodoacetamide in 100 mM ammonium bicarbonate pH 8.0 for 30 min at room temperature and then digested with 25 $\mu g.\mu L^{-1}$ of trypsin solution (Tripsin Porcine, Mass Spectrometry Grade, Promega). In-gel trypitc degradation was performed overnight at 37 °C. The samples were desalting using C18 micro columns (Zip Tip, Millipore, Ireland) to avoid interference in crystallization of the samples with the matrix used in for MALDI-TOF/MS analysis, and were then stored at -20 ^{o}C until subsequent analysis.

Protein identification

The protein identification was performed using matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/MS). The sample solution was applied to the matrix α-cyano-4-hydroxycinnamic acid solution (5 $\mu g.mL^{-1}$ in 50% acetonitrile and 0.1% TFA) onto the MALDI-TOF/MS target (steel plate MTP Anchor Chip TM 600/384 TF, Bruker Daltonics). The MALDI-TOF spectra were

calibrated using trypsin Promega porcine peptide signals and matrix ion signals by Bruker Daltonics flexAnalysis Software; MALDI analysis was performed by a feedback control systems (Ultraflex III MALDI TOF/TOF system, Bruker, Germany). The MALDI-TOF/MS analyses were performed with Reflective Positive Peptide method. Peptide masses were searched against the NCBI and SwissProt database with 0.5 error using the Mascot program (http://www.matrixscience.com) and MASCOT Peptide Mass Fingerprinting database search. The initial search variables for MS analysis allowed a single trypsin missed cleavage, no restriction on protein mass, carbamidomethyl (C) for fixed modifications, oxidation (M) for variable modifications, peptide mass tolerance of 0.5 and the taxonomic search space was restricted to mammalian. The protein identification by MS/MS analyses (MALDI-TOF/TOF) was performed using LIFT method. The peptide ions were searched against the NCBI and SwissProt database with 0.5 error using the Mascot program (http://www.matrixscience.com) and MASCOT MS/MS Ions Search database. The search variables for MS/MS analysis allowed a single trypsin missed cleavage; peptide charge 1+; carbamidomethyl (C) for fixed modifications; oxidation (M) for variable modifications; 0.5 peptide mass tolerance; 0.5 MS/MS tolerance and the taxonomic search space was restricted to mammalian. Identities with probability-based ($p<0.05$) in SwissProt database and scores > 46 were significant to NCBInr.

RESULTS AND DISCUSSION

Proteins from the *Longissimus dorsi* muscle from the Commercial line and Piau breed were separated by 2-DE technology. A total of 2345 spots were included in the statistical analysis. According to differences between pig genetic groups a total of 408 protein spots were changed as validated by ANOVA and *P*-values < 0.05. After verification of individual spots, 35 protein spots were significantly different between the two genetic groups and were selected for protein identification. Among the selected 35 protein spots, six spots were overexpressed in Piau breed; two spots were overexpressed in Commercial line; three spots were present only in Piau breed and 24 spots were present only in Commercial line. Of these, 18 proteins could not be identified due to lack of sequence similarities in the database and 5 proteins could not be extracted from the preparative gels due to low abundance or poor separation. The list of identified

proteins is shown in Table 1 and the representative gels and the identified proteins are presented in Figure 2.

Table 1. Differentially expressed muscle proteins in Commercial line and Piau breed identified by 2-DE and MALDI-TOF/TOF mass spectrometry[1]

Spot no.	Identified proteins[2]	Experimental p*I*/MW[3]	Theoretical p*I*/MW[2]	Matched peptides/% sequence coverage[4]	Mascot score	Accession[5]	Source[2]	Database	Ratio P/C[6]	*P*-value[7]
Structural proteins										
1	Putative beta-actin-like protein 3	6.25/71201	5.91/42331	8/27	67	ACTBM_HUMAN	Homo sapiens	SwissProt	C	0.0017
2	Troponin T, fast skeletal muscle	9.39/41578	5.99/32107	13/38	84	TNNT3_BOVIN	Bos taurus	SwissProt	C	0.0301
Metabolic enzymes										
3	Fructose-bisphosphate aldolase A	8.18/43316	8.30/39851	14/42	51	ALDOA_HUMAN	Homo sapiens	SwissProt	1.21	0.0001
Cellular defense/ Stress proteins										
4	Alpha-crystallin B chain	6.81/25043	6.76/20024	8/24	37	CRYAB_BOVIN	Bos taurus	SwissProt	1.36	0.0001
5	Heat shock protein 27	7.93/64811	7.87/22427	9/33	54	gil662841	Homo sapiens	NCBI	1.39	0.0002
6	Heat shock protein 27	7.48/22428	7.87/22427	17/14	76	gil662841	Homo sapiens	NCBI	C	0.0010

[1]Abbreviations: P = Piau pig; C = Commercial line.
[2]Protein name, theoretical p*I* and MW and accession numbers were derived from database.
[3]MW = molecular weight; p*I* = isoelectric point.
[4]The number of matched peptides in the database search and the minimum coverage of the matched peptides in relation to the full-length sequence.
[5]The database search.
[6]Relative differences in 2-dimensional gels electrophoresis gel spot intensities: Piau breed spot intensity/Commercial line spot intensity. C = spots presents only in Commercial line.
[7] *P*-value from the Student's test.

The structural proteins beta actin and troponin T was identified only in Commercial line. The myofibrillar proteins form about 60% of the total muscle protein. Actin forms the major parts of the thin filament. It consists of globular molecules that, in relatively concentrated salt solutions polymerize to form double helical chains. These form the basis of the thin filaments together with two other proteins, tropomyosin and troponin modulating the contraction of striated muscle. The troponin T belongs to the troponin complex and binds to tropomyosin. Troponin and tropomyosin together make up 10% of the myofibrillar proteins and are involved in the control of muscle contraction by calcium ions (Warriss, 2000). The actin protein is frequently associated with differential expression between breeds (Hollung, at el., 2008; Xu et al., 2009; Murgiano et al., 2010) and age (Hollung et al., 2008; Xu et al., 2012). The actins levels are positively correlated with synthesis of fiber muscle proteins and with muscle growth (Murgiano et al., 2010) and was related to tenderness at lower levels (Ametsch et al.,

2003; Hollung et al., 2008). According to this, the presence of these proteins only in Commercial line cannot indicate the absence in the Piau breed. It may be due to poor separation during electrophoresis in the second dimension.

The fructose-bisphosphate aldolase A is a glycolytic enzyme that catalyzes the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Aldolase A is found in the developing embryo and is produced in even greater amounts in adult muscle. Its expression was shown to be modulated in response to alterations in contractile activity in the chicken, rat and in the rabbit. The overexpression in Piau breed may be due to response to external influences including relative muscle usage, motor neuron activity, energy source availability and hormonal status (Spitz et al., 1998).

The chaperone protein α B-crystallin is considered a member of the class of small heat shock proteins (HSPs). They are associated with the nucleus and are localized in the cytoplasm under normal conditions (Klemenz, et al., 1991). Is has been identified in porcine proteome muscle at higher levels in older animals (Hollung et al., 2008) and reflect the predominant oxidative metabolism (Sayd et al., 2006). The HSP27 also kwon as stress protein, plays a universal role of maintaining the cellular homeostasis and has been shown to respond to muscle disease and after exercise (Xu et al., 2009). Distinct HSPs play different muscle functions. The HSP27 is highly abundant in skeletal muscle and play a role to organize and to protect the myofibrillar structure (Kim et al., 2004; R Lametsch et al., 2006; Sayd et al., 2006; Hollung et al., 2008). Heat-shock protein 27 and α-crystallin were also associated with the organization and protection of the myofibrils by Lametsch (2011). Besides, the expression and modification of these proteins were affected by conditions such as stress. The results indicated that these proteins may affect meat quality by stabilizing the myofibrils postmortem and they could also be useful biomarkers for PSE (Pale, Soft and Exudative) meat or stress. According to Sayd et al. (2006), the α B-crystallin and HSP27 were more abundant in dark muscle samples due to greater amount of oxidative fibers, in muscle of type I fibers and in response to an increase demand in oxidative metabolism, like during sustained exercise. In this way, the overexpression of the α B-crystallin and HSP27 in local Brazilian breed Piau may reflect the oxidative metabolism of fibers muscle. The HSP27 was also identified in Commercial line but with another molecular weight. When the same protein is present in different positions on the 2-DE

gels, it may reflect different isoforms of the proteins. The isoforms can be originated by modifications that influence the charge of the protein or it may also reflect the fragmentation of the protein. When the protein had a molecular weight close to the theoretical calculated that is probably a full-lengh protein (Hollung et al., 2008). The up regulation of HSP27 was associated with glycolytic metabolism in Large White breed. Besides, the HSPs may have an important role in postnatal muscle growth of different breeds (Xu et al., 2009).

In the oxidative metabolism, the muscle fibers require more lipids than carbohydrates as energy source. Its related to IMF content in skeletal muscles (Hocquette et al., 1998) and is more abundant in breeds that have never undergone genetic improvement. This type of metabolism could also be observed in skeletal muscle of Piau breed by the functional annotation of transcripts (Nascimento et al., 2012). In this study, the overexpression of energy metabolism enzymes as NADH dehydrogenase, cytocrome c oxidase and cytocrome b reductase, was elevated in the Piau skeletal muscle compared to the Commercial line. These results suggest that the local Brazilian breed Piau presents a higher oxidative capacity than the Large White and Landrace breeds. Proteomic study related to oxidative metabolism in local breeds have been reported by Xu et al. (2009) in Meishan Chinese breed. The proteomics-based investigation of different pig breeds in *Longissimus* muscle allowed the identification of 14 proteins, including energy metabolic enzymes, myofibrillar proteins, cell defense and stress related protein. The proteins related to oxidative metabolism were cytosolic glycerol-3-phosphate dehydrogenase; and ATPase β chain. Li et al. (2013) comparing the protein expression of *Longissimus* muscle between the local Chinese pig breed Lantang and Landrace pig, identified 18 differentially expressed proteins. The proteins COX5A (similar to mitochondrial cytochrome-c oxidase subunit) and the ATP5B (mitochondrial ATP synthase, H + transporting F1 complex β subunit) were associated to oxidative capacity to the skeletal muscle.

The use of RNAholder® for tissue preservation is not the usual method to perform sample preparation in proteomic studies. The similar reagent RNAlater®, functions by rapidly infiltrating cells with a high concentration of ammonium sulfate, causing a mass precipitation of cellular proteins while cellular structure remains intact (Lader, 2001). Despite the use of salting media, usually kwon to interfere in two-dimensional electrophoresis, the identification of proteins from porcine muscle by 2-DE

was possible in present study and allowed to concluded about differences between both pig genetic groups evaluated. Previously study (not published) comparing the two methods of tissue preservation in porcine muscle was performed and the gels from RNAholder® presented more proteins than the frozen tissue storage method. Although the use of RNAholder® to preserve samples may have prejudice focusing and protein identification possibly due to presence of salt, the use of the material maintained in this medium is extremely valuable because it allows transcriptional analysis exactly in the same material. It guarantees the supplementary information to evidence different metabolic events in the two different pig genetic groups. Thus, new proteomic studies must be realized to improve quality of the protein isolation in the gels, to deplete of high abundance proteins, for application of narrow pH gradient to allow separation of proteins by using IPG strips pH 3-6, pH 5-8, or pH 7-19, with linear or non-linear pH gradient on the strips.

CONCLUSIONS

The proteomic profiles of porcine muscle can be performed using RNAholder® solution as tissue storage and preservation. The identified proteins pointed to different metabolism between divergent genetic groups. The local Brazilian breed Piau indicated oxidative metabolism instead glycolytic metabolism observed in Commercial line. The poor separation of the protein spots and the low abundance of identified proteins can indicate some interference during the 2-DE electrophoresis. New trials should be realized to improve the information between these divergent genetic groups .

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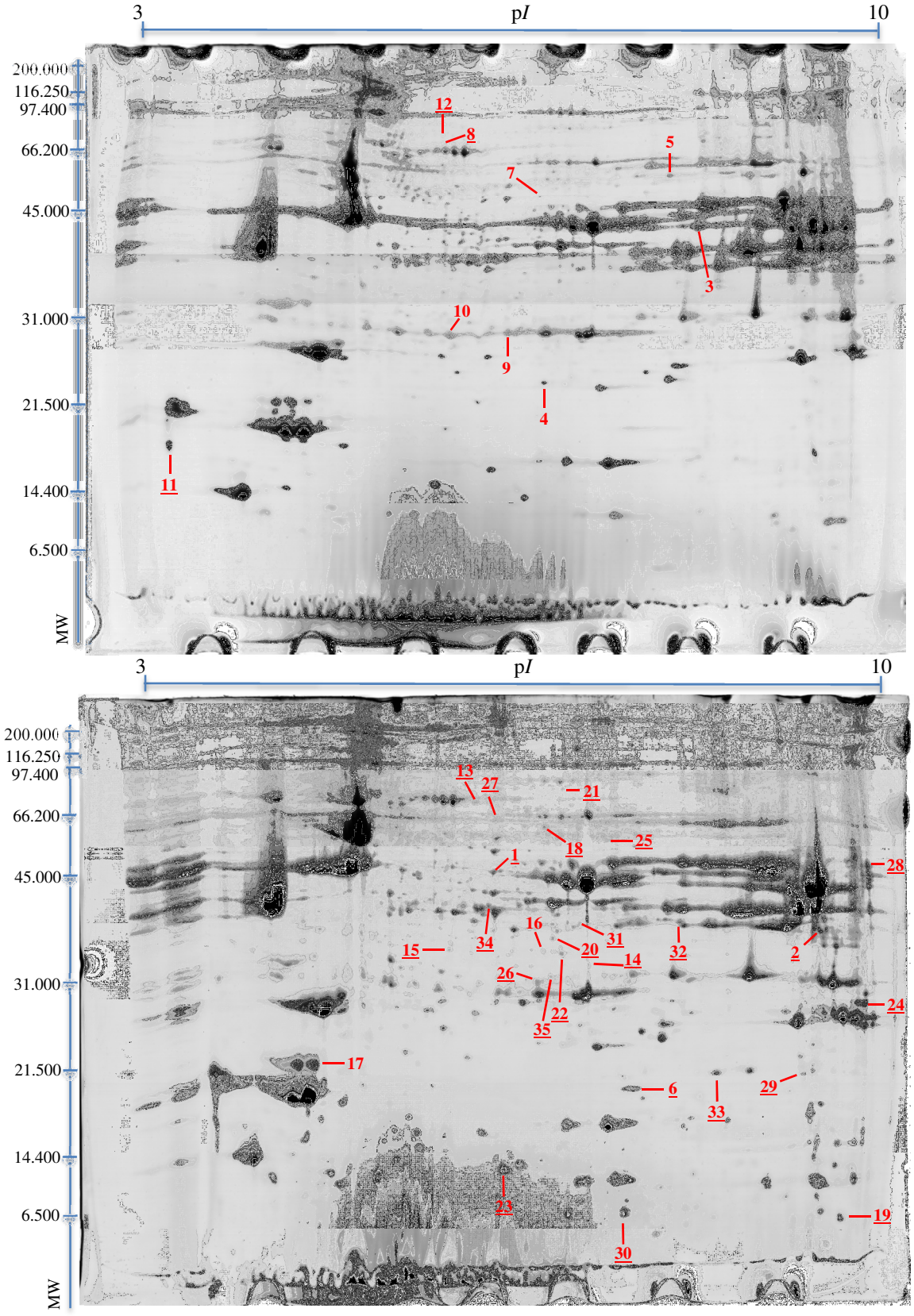

Figure 2. Representative 2-DE image of *Longissimus dorsi* muscle sample from (A) Piau breed and (B) Commercial line. Proteins are separated by pH 3-10 in the first dimension and 12.5% SDS-PAGE in the second dimension. The overexpressed proteins are marked in red with respective numbers within groups. The proteins present only in Piau breed or Commercial line are marked in red and subscribed.

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GENERAL CONCLUSIONS

The use of RNAholder® was effective to identify proteins for proteomic purposes. The amount of proteins were superior than the standard method using liquid nitrogen suggesting that this solution are able to separate proteins to RNA molecules inside the cell better than frozen the tissue.

The comparison between *Longissimus dorsi* muscle from local Brazilian breed Piau and Commercial line using RNAholder® method of tissue preservation allowed identifying structural proteins and proteins related to defense or stress proteins. The local Brazilian breed Piau indicated oxidative metabolism instead glycolytic metabolism observed in Commercial line. The poor separation of the protein spots and the low abundance of identified proteins can indicate some interference during the 2-DE electrophoresis or the large amount of proteins loaded in the strips before the IEF process. New trials should be realized to improve the information between these divergent genetic groups.