Ractopamine-induced changes in sarcoplasmic proteome profile of post-rigor pork *semimembranosus* muscle

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Abstract

Ractopamine is a beta-adrenergic agonist that increases leanness and carcass weight in finishing pigs. Our previous study observed that dietary ractopamine increased the abundance of several glycolytic enzymes in the sarcoplasmic proteome of post-rigor pork *longissimus thoracis* muscle. Pork *semimembranosus* is an economically important muscle and demonstrates differences in biochemistry compared with *longissimus thoracis*. Nonetheless, the effects of ractopamine on sarcoplasmic proteome of *semimembranosus* have not been evaluated yet. Therefore, this study examined the influence of ractopamine on sarcoplasmic proteome of post-rigor pork *semimembranosus*. Analyses of sarcoplasmic proteome of *semimembranosus* muscles from control (CON; diet without ractopamine) and ractopamine-fed (RAC; 7.4 mg/kg for 14 days followed by 10.0 mg/kg for 14 days) barrows revealed that haemoglobin subunit beta, alpha-crystallin B, and titin fragments were over-abundant in CON. In contrast, myosin light chain 1/3 and tripartite motif-containing protein 72 were over-abundant in RAC. The low abundance of haemoglobin subunit beta and alpha crystallin B in RAC could be attributed to fibre type shift (from oxidative to glycolytic) in response to ractopamine. The over-abundance of MLC 1/3 and tripartite motif-containing protein 72 in RAC could be due to the increased myofibrillar protein synthesis and muscle mass in ractopamine-fed pigs. Dietary ractopamine decreased the abundance of sarcoplasmic proteins involved in oxygen transport and chaperone activity, but increased the abundance of proteins involved in muscle contraction and plasma membrane repair in pork *semimembranosus* muscle.

Keywords: Pork, ractopamine, sarcoplasmic proteome, *semimembranosus*
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Introduction

The consumer demand for meat as a source of high quality animal protein is increasing (Lusk, 2013) and the applications of growth technologies in livestock production play a critical role in achieving global food security (Johnson *et al*., 2013; Dunshea *et al*., 2016). Beta-adrenergic agonists are oral growth promotants approved for use in food animals (Johnson *et al*., 2013). Ractopamine is a beta-agonist approved for use in swine industry to increase growth rate, feed efficiency, carcass yield, and leanness in finishing pigs (Apple *et al*., 2007; Dunshea, 2012; Pompeu *et al*., 2017) in more than 25 countries, including the United States, Canada, Australia, Mexico, Philippines, Brazil, sand South Korea (AMI, 2012). Dietary ractopamine increases leanness (Boler *et al*., 2011) and carcass weight (Stites *et al*., 1991; Armstrong *et al*., 2004; Fernandez-Duenas, 2008; Needham & Hoffman, 2015) in pigs, and the increased carcass weight is a consequence of the increase in myofibrillar protein synthesis (Adeola *et al*., 1992).

Proteomic tools are applied to characterize the role of muscle proteome in meat quality (Suman *et al*., 2014; Gobert *et al*., 2014; Joseph *et al*., 2015; Schilling *et al*., 2017). The sarcoplasmic proteome, comprising 30% of total muscle proteins, consists of enzymes and proteins critical to metabolism in live skeletal muscle and biochemical processes influencing the properties of post-rigor muscle as food (Scopes, 1970). In this regard, our recent study examined the effect of ractopamine on the sarcoplasmic proteome of post-rigor pig

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longissimus thoracis and reported that ractopamine increased the abundance of glycolytic enzymes and chaperones (Costa-Lima *et al*., 2015).

Longissimus thoracis et lumborum and *semimembranosus* are two economically important pork muscles (Jones & Burson, 2000), which demonstrate differences in biochemical properties, such as pH, mineral content, fatty acid profile, transcriptome, and endogenous antioxidants (Huff-Lonergan *et al*., 2002; Purchas *et al*., 2009; Herault *et al*., 2014). Therefore, it is possible that the ractopamine-induced proteome changes in post-rigor pork *longissimus thoracis* could be different from those in *semimembranosus*. Nonetheless, the influence of ractopamine on sarcoplasmic proteome of pork *semimembranosus* is yet to be evaluated. Therefore, the aim of this study was to examine the effect of ractopamine on sarcoplasmic proteome of post-rigor pork *semimembranosus* muscle.

Materials and Methods

The animal care protocol (Number: 2011A00000141) was approved by the Institutional Animal Care and Use Committee at The Ohio State University (Columbus, OH, USA). Purebred Berkshire pigs were used in this study because they are often used in branded pork programs (McMullen, 2006). Two-hundred purebred Berkshire pigs (barrows and gilts; average initial bodyweight of 68.9 kg) were used as previously described (Bohrer *et al*., 2013). All animals were raised under similar conditions at The Ohio State University Western Agricultural Research Station (South Charleston, OH, USA). The pigs were stratified based on initial body weight over 2 blocks and were housed in mixed-sex pens, and pens served as replicates. Each block consisted of 10 pens (5 pens x 2 dietary treatments). Within each dietary treatment, 4 pens had 6 barrows and 4 gilts, whereas 1 pen contained 5 barrows and 5 gilts. Overall pen size was 16.25 m^2 (including 3.9 m^2 slatted-floor area), with each animal receiving approximately 1.63 m^2 floor space. Each pen had a double-nipple water drinker and a 4-hole single-sided box feeder that provided a total of 1.22 m of linear feeder space. Pigs were housed in a curtain-sided and naturally ventilated barn and were provided *ad libitum* access to feed and water throughout the finishing.

Pigs were provided a 14 d adaptation period prior to the start of the treatment diets. Within each block, pigs in 5 pens were finished on a step-up ractopamine (Paylean; Elanco Animal Health, Greenfield, IN, USA) diet (RAC; 171 g/kg crude protein and 10.4 g/kg total lysine) with 7.4 mg/kg ractopamine for 14 d followed by 10 mg/kg ractopamine for the last 14 d prior to slaughter. The pigs in the other 5 pens within the block were finished on a control diet (CON; 131 g/kg crude protein and 7.6 g/kg total lysine) with 0 mg/kg ractopamine. Diets were analysed to ensure ractopamine inclusion levels were within acceptable tolerances (75 to 125%) of the claim for each diet.

Previous research documented that the effect of ractopamine on color and texture of ready-to-eat pork products (i.e. frankfurters) is sex-specific (Costa-Lima *et al*., 2014). Therefore, to avoid any potential effect of sex on skeletal muscle proteome, only barrows were selected for proteomic investigation. One barrow was randomly selected from each of the 10 pens in CON (104 kg average body weight) and RAC (107 kg average body weight) at the end of the 28-day feeding period, and these 20 pigs were transported to The Ohio State University Meat Science Laboratory. This approach provided 10 replicates (n = 10) for proteome analysis, of which nine were essentially the same animals used in our previous work (Costa-Lima et al., 2015) on the effect of ractopamine on proteome of pork *longissimus thoracis*. The pigs were kept overnight in lairage with free access to water, but with no access to feed, before being humanely slaughtered. The carcasses were chilled for 24 h at 4 °C before fabrication. Fresh hams from the right side of the carcasses were collected. From the centre of the inside portion of the hams (IMPS # 402F), a 2.54-cm thick slice of *semimembranosus* muscle was removed. The muscle samples were individually vacuum-packaged, frozen at –80 °C, and transported in dry ice to the University of Kentucky (Lexington, KY, USA) for proteome analysis.

Sarcoplasmic proteome was extracted according to the previously described method (Joseph *et al*., 2012). Five grams of frozen muscle samples were homogenized in 25 mL ice-cold extraction buffer (40 mM Tris, 2 mM EDTA, and pH 8.0). The homogenate was centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant (sarcoplasmic proteome extract) was filtered and utilized. The protein concentration of the sarcoplasmic proteome extract was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). An aliquot corresponding to 900 µg of protein was mixed with rehydration buffer (optimized to 7 M urea, 2 M thiourea, 40 g/L CHAPS, 20 mM DTT, 5 g/L Bio-Lyte 5/8 ampholyte (Bio-Rad, Hercules, CA, USA), and 0.01 g/L Bromophenol blue) and was loaded onto immobilized pH gradient (IPG) strips (pH 5–8, 17 cm). Gels were subjected to passive rehydration for 16 h, and then subjected to first-dimension isoelectric focusing (IEF) in a Protean IEF cell system (Bio-Rad, Hercules, CA, USA) applying a linear increase in voltage initially and a final rapid voltage ramping to reach a total of 80 kVh. Subsequently, the IPG strips were equilibrated in SDS-containing buffers, first with equilibration buffer I (containing 6 M urea, 0.375 M Tris-HCl, pH 8.8, 20 g/L SDS, 200 g/L glycerol, 20 g/L DTT) for 15 min, followed by equilibration buffer II (containing 6 M urea, 0.375

M Tris-HCl, pH 8.8, 20 g/L SDS, 200 g/L glycerol, 25 g/L iodoacetamide) for 15 min. The proteins were resolved in the second dimension on 13.5% SDS–PAGE (38.5:1 ratio of acrylamide to bis-acrylamide) using Protean II XL system (Bio-Rad, Hercules, CA, USA). The gels were stained using Colloidal Coomassie Blue for 48 h and destained until background was cleared. Samples of both treatments (CON and RAC) were run under the same conditions (2 gels/pig), resulting in a total of 40 gels.

Digital images of the stained gels were captured using Versa Doc (Bio-Rad, Hercules, CA, USA) and analysed using PDQuest (Bio-Rad, Hercules, CA, USA). Images were first subjected to automatic spot detection and matching optimized by the aid of landmark protein spots, and the matched spots were normalized (Meunier *et al*., 2005). A total of 182 spots were identified and evaluated in the image analyses. For each spot in a given sample, spot quantity values in duplicate gels were averaged for statistical analysis (Joseph *et al*., 2012). A protein spot was considered to be differentially abundant when it was associated with 1.5-fold intensity difference and 5% significance ($P < 0.05$) level in a pairwise Student's t-test (Joseph *et al.,* 2012).

The spots exhibiting differential abundance between the treatments were excised from the gel using pipet tips, placed in microtubes for destaining by two 30 min washes with 50 mM NH4HCO3/500 g/L CH3CN, vortexed for 10 min, and dried in a vacuum centrifuge. The respective spot was excised from the counterpart treatment to confirm the match. Proteins in the gel fragment were reduced by reaction with 10 mM DTT in 50 mM NH4HCO3 solution and incubation at 57 °C for 30 min. The supernatant was discarded, and the proteins (present at the gel piece) were alkylated by addition of 50 mM NH4HCO3 containing 50 mM iodoacetamide and incubated for 30 min at 25 °C without exposure to light. Further, the gel piece was washed twice with 50 mM NH4HCO3 and once with CH3CN, and then partially dried in a vacuum centrifuge. The dried gel piece was rehydrated with a solution of 40 mM NH4HCO3/90 g/L CH3CN, containing 20 mg/L of proteomic grade trypsin (Sigma, St. Louis, MO, USA) on ice for 1 h. An additional volume of 40 mM NH4HCO3/90 g/L CH3CN was added to cover the sample, and the microtube was incubated for 18 h at 37 °C. Peptides were extracted from the gel piece in 0.1 % trifluoroacetic acid by sonication for 10 min followed by vortexing for 10 min, and then the extraction was repeated using a solution on 500 g/L acetonitrile containing 1 g/L trifluoroacetic acid. The extracts were combined, and the volume was reduced to remove most of the acetonitrile. Peptide extracts were desalted and concentrated using solid phase extraction using 1 mm of Empore C-18 (3M, St. Paul, MN, USA) packed in a 0.1 to 10 µL pipet tip (Sarstedt, Newton, NC, USA). The peptides were eluted in 5 µL of 500 g/L CH3CN/0.1% trifluoroacetic acid.

An aliquot of 0.3 µL of the desalted peptide extract was spotted onto an Opti-ToF 384 well insert (AB Sciex, Foster City, CA, USA) with 0.3 µL of 5 g/L α-cyano-4-hydroxycinnamic acid (Aldrich, St. Louis, MO, USA) in 50 g/L CH3CN/1 g/L trifluoroacetic acid. Crystallized samples were washed with cold 1 g/L trifluoroacetic acid and were analysed using a 4800 MALDI TOF-TOF Proteomics Analyzer (AB Sciex, Foster City, CA, USA). An initial MALDI MS spectrum was acquired for each spot (400 laser shots/spectrum), and a maximum of 15 peaks with a signal-to-noise ratio of more than 20 were automatically selected for MS-MS analysis (1000 laser shots/spectrum) by post-source decay. The peak lists from the MS-MS spectra were submitted for similarity search in the National Center for Biotechnology Information (NCBI) database using Protein Pilot version 4.0 (AB Sciex, Foster City, CA, USA) employing relevant search parameters (Search type: identification; Enzyme: trypsin; Database: porcine NCBI nr; Search effort: thorough; Unused cut off > 1.30, 95% confidence) to identify the proteins. A protein was assigned to be identified if it had at least 2.00 unused cut off value and at least two peptides were identified with 99% confidence.

Results and Discussion

Gel image analyses identified 6 differentially abundant protein spots (Figure 1). Four protein spots were over-abundant ($P < 0.05$) in CON, whereas 2 spots were over-abundant ($P < 0.05$) in RAC. The identity of these proteins and their functional roles are listed in Table 1.

Haemoglobin subunit beta was over-abundant in the CON group (Table 1). Haemoglobin is a tetrameric heme protein comprising 2 alpha (141 residues each) and 2 beta (146 residues each) subunits, and is the major oxygen carrier protein. Based on metabolism, skeletal muscles fibres are classified into 3 types, namely oxidative, oxido-glycolytic, and glycolytic (Peter *et al*., 1972). Ractopamine functions as a repartitioning agent in skeletal muscles and activates beta-adrenergic receptors, leading to fibre type shifting from oxidative to glycolytic, thereby promoting glycolytic metabolism (Aalhus *et al*., 1992; Depreux *et al*., 2002; Gunawan *et al*., 2007). The decreased abundance of haemoglobin in the RAC group could possibly be attributed to this shift in metabolism from oxidative to glycolytic metabolism in response to ractopamine feeding. Glycolytic metabolism has lower oxygen demand compared to oxidative, which minimizes the necessity for haemoglobin to transport oxygen to the skeletal muscles.

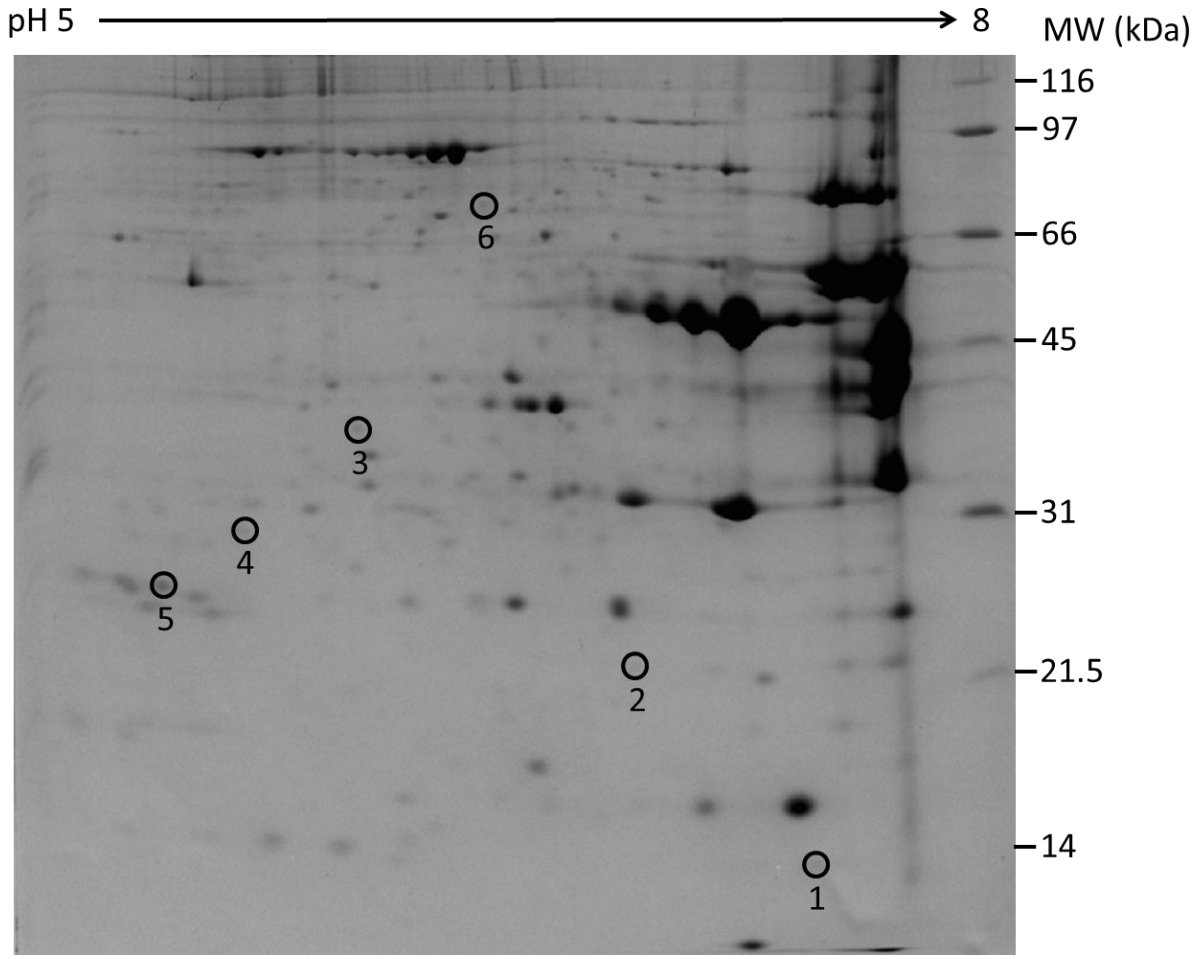

Figure 1 Coomassie-stained two-dimensional gel of the sarcoplasmic proteome extracted from post-rigor pork semimembranosus muscle. Six proteins spots differentially abundant in control and ractopamine-fed pigs are encircled and numbered

Haemoglobin is a minor contributor to color of fresh meats (Mancini & Hunt, 2005; Suman & Joseph, 2013) and previous proteomic investigation on pork *semimembranosus* sarcoplasm reported that haemoglobin was of greater abundance in dark muscles than in light muscles (Sayd *et al*., 2006). Several previous studies observed that fresh meat from pigs fed ractopamine had lower a* values (redness) than meat from animals raised on control diets (Uttaro *et al*., 1993; Armstrong *et al*., 2004; Carr *et al*., 2005a; 2005b; Apple *et al*., 2008). The decreased abundance of haemoglobin subunit beta in the RAC group suggested that ractopamine feeding may cause a decrease in *a** value of pork (Aalhus *et al*., 1990), attributing to the shift from intermediate fibres to white fibres (Depreux *et al.,* 2002; Gunawan *et al*., 2007).

Spots 3 and 4 (Fig. 1) were identified as titin fragments and were over-abundant in the CON group (Table 1). Titin is the largest protein in mammals and has a molecular weight of approximately 3,800 kDa; it is the third most abundant myofibrillar protein (Labeit *et al.,* 1997). Although titin is a myofibrillar protein, the appearance of its fragments in sarcoplasm could be due to post-mortem proteolysis (Taylor *et al*., 1995). The low sequence coverage of titin (Table 1) could be attributed to multiple proteases, such as calpain proteinases (Huff-Lonergan *et al*., 2010; Lian *et al*., 2013), lysing the protein into small fragments and the large size (more than 34,000 amino acids) of titin molecules. Degradation of titin has been reported to increase meat tenderness (Huff-Lonergan *et al*., 1995), and the over-abundance of titin fragments in CON could be due to decreased proteolysis in the RAC group. In support, dietary ractopamine has been reported to decrease proteolytic activity in pork *longissimus* (Xiong *et al*., 2006).

Myosin light chain (MLC) 1/3 was over-abundant in the RAC group (Table 1). These findings are also supported by Costa-Lima *et al.* (2015), who reported a greater abundance of MLC 1/3 in *longissimus thoracis* of ractopamine-fed pigs than in pigs fed a control diet. Myosin is a myofibrillar protein, and the hexameric myosin molecule consists of 2 heavy chains (MHC), 2 regulatory light chains (RLC), and 2 alkali light chains

(MLC 1, MLC 3) in fast-twitch skeletal muscles (Frank & Weeds, 1974). Two alkali light chains (MLC 1 and MLC 3) observed in the sarcoplasmic fraction in the present study could be the products of proteolytic cleavage, which releases the light chain sub-units from the actomyosin complex to sarcoplasm (Gerlemann *et al*., 2014).

Table 1 Identity and functional roles of differentially abundant sarcoplasmic proteins in post-rigor *semimembranosus* muscles of ractopamine-fed Berkshire barrows

Spot [a]	Protein	Accession No:	ProtScore/ Matched peptides	Sequence Coverage (%)	Function	Over-abundant treatment [b]	Spot ratio
1	Haemoglobin subunit beta	P02067	18.00/10	70.1	Oxygen transport	CON	1.64 [c]
2	Alpha-crystallin B chain	Q7M2W6	8.00/5	41.7	Chaperone activity	CON	1.82 [c]
3	Titin fragment	350593665	7.81/5	1.6	Structural protein	CON	1.59 [c]
4	Titin fragment	350593665	6.00/3	1.6	Structural protein	CON	1.61 [c]
5	Myosin light chain 1/3	A0JNJ5	11.89/8	45.4	Muscle contraction	RAC	1.59 [d]
6	Tripartite motif-containing protein 72	335284311	19.20/9	29.1	Cell membrane repair	RAC	1.52 [d]

[a] Spot number refers to the numbered spots in gel image (Fig. 1). For each spot, parameters related to protein identification are provided (UniProt accession number; ProtScore and number of matched peptides; sequence coverage of peptides in tandem mass spectrometry)
[b] CON = 0 mg/kg ractopamine in finishing diet for 28 days; RAC = 7.4 mg/kg ractopamine in finishing diet for 14 days followed by 10.0 mg/kg for 14 days
[c] Spot ratio of CON/RAC
[d] Spot ratio of RAC/CON

Previous studies documented that dietary ractopamine increased myofibrillar protein synthesis in pork *longissimus* (Adeola *et al.,* 1992). Furthermore, Helferich *et al.* (1990) observed an increase in the MLC 1/3 RNA expression in ractopamine-fed porcine *longissimus* indicating protein accretion and increase in muscle mass. The greater abundance of MLC 1/3 in the RAC group than in the CON group observed in the present study could be due to the increased myofibrillar protein synthesis in response to ractopamine. Several proteomic investigations have been undertaken to identify the relationship between myosin light chains and meat quality. A positive correlation between MLC 1 and shear force has been observed in pork *longissimus* (Hwang *et al*., 2005). Furthermore, in beef *longissimus thoracis*, MLC 1 was of greater abundance in tough meat than in tender meat (Bjarnadottir *et al*., 2012).

Tripartite motif-containing protein 72 plays a critical role in plasma membrane repair and was over-abundant in the RAC group (Table 1). The cellular mechanism for plasma membrane repair and remodelling, to maintain cellular homeostasis in response to various physiological and pathological conditions, is conserved across eukaryotes (Han & Campbell, 2007). Tripartite motif-containing protein 72 (mitsugumin-53) is an essential component involved in plasma membrane repair in mouse skeletal muscle (Cai *et al*., 2009). It is well documented that dietary ractopamine increases carcass weight in finishing pigs (Stites *et al*., 1991; Armstrong *et al*., 2004; Fernandez-Duenas *et al*., 2008). Furthermore, an increase in the weight of inside ham (consisting of *semimembranosus* muscle) was observed in ractopamine-fed pigs (Boler *et al*., 2011). The increased muscle growth due to ractopamine could possibly demand increased levels of plasma membrane repair proteins to maintain homeostasis. Therefore, the over-abundance of tripartite motif-containing protein 72 in RAC could be attributed to the increased muscle mass accretion in ractopamine-fed animals (Bohrer *et al*., 2013).

Conclusion

The results of the present study suggest that dietary ractopamine influences the abundance of proteins involved in oxygen transport, chaperone activity, and plasma membrane repair in sarcoplasmic proteome of pork *semimembranosus* muscle. This change in sarcoplasmic proteome profile may be primarily due to the previously documented muscle fibre type shift (from oxidative to glycolytic) and the increased muscle growth in response to ractopamine feeding. Additionally, the differences between the effects of

ractopamine on sarcoplasmic proteome in *semimembranosus* in the present study and those previously reported in *longissimus thoracis* (Costa-Lima *et al*., 2015) warrant further studies on muscle-specific effects of this beta agonist in pork.

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Authors' Contributions

Conception and design – SPS, DDB, & XL; Data collection and analyses– JW, BMB, MNN, SL, & CMB; Drafting of paper – JW & MNN; Critical revision and final approval of version to be published – SPS.

Conflict of Interest Declaration

The authors declare that they have no affiliations with any organization or entity with any financial or non-financial interest that could bias the subject matter and outcomes discussed in this manuscript.

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