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Comparison of two muscle fibre staining techniques and their relation to pork quality traits

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Abstract: This study compared two histochemical staining methods of muscle fibres and evaluated their relationship with the meat quality traits of two high-value porcine muscles. Immunohistochemical (IHC) and adenosine triphosphatase (ATPase) staining was used to assess the cross-sectional area and proportion of fibre-types I, IIa, IIx and IIb in the samples of *longissimus lumborum* (LL) and *psoas major* (PM) muscles collected one-hour *post-mortem* from 25 crossbred pigs [Large White$_{Sire}$ × (Landrace × Large White$_{Dam}$)] at an average age of 152 days. Muscles differed in all fibre parameters, except the proportion and relative area of type IIx fibres. The LL muscle exhibited greater fibre cross-sectional areas of all fibre types, higher proportions of type IIb/IIB, and lower proportions of I and IIa fibres than the PM muscle in both staining techniques. These two muscles also differed marginally in moisture, crude protein and intramuscular fat content. The PM muscle showed a low correlation between fibre types and chemical composition, but the LL muscle showed moderate correlations between fibre CSA and area composition for moisture and ash content. After IHC staining, an increase in LL eye muscle area and drip loss were correlated with lower proportions of type I fibres, while a greater proportion of type IIx fibres resulted in increased LL eye muscle area and moisture content. Furthermore, a higher CSA of all fibre types in the LL decreased redness (a*) and moisture content of the muscle. Results showed that IHC is more appropriate than ATPase staining for the assessment of relationships between muscle fibre parameters and meat quality traits in pigs.

Keywords: ATPase activity; histology; immunohistochemistry; muscle fibre type; myosin heavy chain isoform

Muscle fibres are the basic functional metabolic unit of skeletal muscles, and depending on the location and function of the muscle within an animal, muscle fibre type composition varies considerably (Realini et al. 2013). Muscle fibres are traditionally classified as type I (slow-twitch oxidative), IIA (fast-twitch oxidative glycolytic) and IIB (fast-twitch glycolytic) (Gauthier 1969; Brooke and Kaiser 1970; Ashmore and Doerr 1971). The molecular basis of this typology resides in the poly-

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morphism of myosin heavy chains (MyHC) (Weiss et al. 1999). Four major MyHC isoforms (I, IIa, IIx, and IIb) have been described in the skeletal muscle of adult mammals, but not all of them are expressed in the muscles of large livestock animals. However, all these isoforms are expressed in pig skeletal muscles during postnatal development (Lefaucheur et al. 2004; Lefaucheur 2010; Fazarinc et al. 2013). Fibre typing according to MyHC isoforms is feasible thanks to immunohistochemical (IHC) analysis, using monoclonal antibodies. This analysis allows the identification of four main muscle fibre types and their hybrid types (co-expression of two or more MyHC isoforms in one fibre) (Kim et al. 2013).

Classical histochemical staining methods, such as staining with ATPase, succinate dehydrogenase (SDH) or NADH-tetrazolium reductase, focus on the contractile and metabolic characteristics of muscle fibres, and thus identify fibre types based on selected parameters, such as oxidative and glycolytic capacities, or the stability of actomyosin ATPase to pH preincubation (Choi and Kim 2009). These classification systems based on stains for enzymes involved in oxidative metabolism and ATPase activity may be suboptimal for fast fibre type classification, as they distinguish only between two fast fibre types (i.e. IIA and IIB) and can also be incompatible between staining techniques (Klont et al. 1998). According to previous studies (Essen-Gustavsson and Lindholm 1984; Fernandez et al. 1995), the proportion of fast fibre types can differ by approximately 7–20% depending on the staining technique used. On the contrary, the slow fibre types (I) correspond well between these studies, and also in comparison with results obtained using IHC staining techniques (Klont et al. 1998; Behan et al. 2002). Despite some disadvantages, such as dependence on the enzyme activities, pH, temperature, and sampling promptly *post-mortem*, these methods have been widely used for the evaluation of muscle fibre composition in relation to meat quality traits within various animal species, including pigs, cattle, poultry, rabbits, nutrias and fish (Joo et al. 2013; Chodova et al. 2016; Listrat et al. 2016; Bogucka et al. 2018; Nemecek et al. 2019; Song et al. 2020). On the other hand, IHC analysis can distinguish between three subtypes of fast fibres (IIa, IIx and IIb) as well as hybrid fibres, compared to the two subtypes identifiable with ATPase staining [IIA and IIB; Klont et al. (1998); Lefaucheur (2010)].

Muscle fibre characteristics such as fibre density, cross-sectional area or fibre type proportion influence the meat quality traits of pork, including water-holding capacity, colour or texture (Lefaucheur 2010; Joo et al. 2013; Bogucka and Kapelanski 2016). However, not many studies focus on the relationships between muscle fibre types classified according to MyHC isoforms and fresh meat quality traits. Furthermore, there are few comparisons between staining methods used to identify and quantify muscle fibre types, and their subsequent conclusions regarding their relationship to meat quality, because these studies usually use one type of staining method or a combination of diverse methods (Candek-Potokar et al. 1999; Chang et al. 2003; Realini et al. 2013; Kim et al. 2014). Thus, the objective of this study was to compare two distinct staining methods of muscle fibres and assess the relationship between these fibre type parameters and meat quality traits of porcine *longissimus lumborum* and *psoas major* muscles.

MATERIAL AND METHODS

Animals, husbandry and slaughter

Twenty-five entire male pigs [Large White$_{Sire}$ × (Landrace × Large White$_{Dam}$)] were used in this study, carried out at the experimental station of Czech University of Life Sciences Prague. All procedures described in this experiment were conducted after obtaining the approval by the Local Ethics Commission, case number 02/2017 (CZ21038206). At the start of the study, the animals were 26 days old and their average weight was 7.2 ± 1.4 kg. The pigs were allotted to eight pens, with three or four animals per pen, and fed dry complete feed mixtures (CFM) *ad libitum* during the growing/fattening period of 124 days, as part of a larger feeding experiment. Briefly, three diets were formulated with a gradual transition according to the age and weight of animals as follows: Phase 1 (from 12 kg of body weight; BW) – 13.89 MJ/kg digestible energy (DE); 17.2% crude protein (CP); 1.12% lysine; Phase 2 (BW 45 kg) – 13.59 MJ/kg DE; 16.1% CP; 1.02% lysine and Phase 3 (BW 74 kg) – 13.35 MJ/kg DE; 14.9% CP; 0.91% lysine. The average daily gain of the animals over the entire feeding experiment was 804.35 g/day and the daily feed intake was 1 673 g/day. The pigs were slaughtered at 105.5 ± 7.6 kg, using electri-

https://doi.org/10.17221/23/2020-CJAS

cal stunning and dressed according to routine commercial procedures. All pigs were slaughtered on the same day. Muscle samples for histochemical analysis were taken within 1 h after slaughtering, from the *longissimus lumborum* (LL) muscle at the level of the last rib and from the cranial part of the *psoas major* (PM) muscle. Muscle pH was determined in the LL muscle at 45 min *post-mortem* (pH_{45}), using a portable pH meter (pH 330i/set; WTW GmbH, Weilheim, Germany) at the 13th and 14th thoracic vertebrae. Carcasses were then cooled at 4 °C for 24 h.

Assessment of physicochemical meat quality

At 24 h *post-mortem*, the LL muscle was removed (13th thoracic to 1st lumbar vertebra), and the whole PM muscle was removed from the carcasses. To assess the LL eye muscle area, photographs were taken of the transverse muscle cuts, along with an indication of scale, and the images were evaluated using NIS-Elements AR 3.2. (Nikon Instruments Europe B.V., Amsterdam, Netherlands). Meat surface colour (CIE Lab*) was measured on the LL muscle after 10 min of blooming, using a Minolta CM-700d colorimeter (Konica Minolta, Osaka, Japan). Samples for drip loss measurements were taken from the LL muscle and evaluated by suspending a steak of each muscle in a plastic bag for 24 h at 4 °C, according to Honikel (1998). The following day (48 h *post-mortem*), steaks were cut from the LL muscles (±100 g) and a temperature probe was inserted into the middle of one muscle steak before cooking. Samples were placed into individual plastic bags, and then into a water bath (80 °C) until an internal temperature of 75 °C was reached. Rectangular samples were cut from the raw and cooked muscles (6 × 1 × 1 cm) and Warner-Bratzler shear force values (WBSF; N) were determined on six samples per muscle using an Instron Universal Texture Analyzer 3342 (Instron, Norwood, MA, USA) fitted with a standard Warner-Bratzler blade (triangular hole; 20 N load cell; 200 mm/min crosshead speed; 20 points/s sampling rate).

Muscle samples for chemical analysis of the LL and PM muscle were stored at −80 °C for two weeks. The samples were then thawed, homogenised and subjected to chemical analysis for moisture [AOAC (2005); method 950.46), crude protein content using the Kjeldahl method (AOAC 979.09; KjelFlex K-360, Büchi, Flavil, Switzerland), intramuscular fat (IMF) according to Soxhlet (ISO 1444 Meat and Meat Products – Determination of Free Fat Content) using ether as the solvent (SER 148, VELP Scientifica, Usmate, Italy) and ash content (AOAC 942.05; Ht40AL oven, LAC, Rajhrad, Czech Republic).

Enzymatic immunohistochemical analysis

The LL and PM muscle samples collected 1 hour *post-mortem* were cut into 0.5 × 0.5 × 2.0 cm pieces, immediately frozen in isopentane cooled with liquid nitrogen and stored at −80 °C until analysis. Transverse serial muscle sections of 12 µm were cut from the frozen muscle samples using a Leica CM1850 cryostat (Leica Microsystems, Nussloch, Germany) at −20 °C, and mounted onto glass slides.

To show the expression of MyHC isoforms, four monoclonal antibodies: BA-D5 (specific to MyHC I), BF-35 (MyHC I/IIa), BF-F3 (MyHC IIb) and SC-71 (MyHC IIa/IIx) were purchased from Developmental Studies Hybridoma Bank (DSHB, University of Iowa) in accordance with the methodology of Fazarinc et al. (2013), Realini et al. (2013) and Kim et al. (2014). Serial muscle sections were incubated with the primary antibodies overnight at 4 °C in a humidified box. After incubation, the sections were washed, reacted with peroxidase-conjugated secondary antibody and visualised by incubating the sections in Tris-HCl buffer solution with diaminobenzidine tetrahydrochloride. Muscle fibres were classified into types I, IIa, IIx and IIb according their reaction with specific antibodies (Figure 1). Hybrid fibres which expressed more than one MyHC isoform were classified as one of the pure fibre types according to their reaction intensity with the respective monoclonal antibody.

For histochemical differentiation of type I, IIA and IIB fibres, the consecutively sliced muscle samples of LL and PM were mounted onto two glass slides and stained using the methodology described by Brooke and Kaiser (1970), after acid (pH 4.6) and alkaline (pH 10.3) preincubation (Figure 2). Images of the muscle samples were obtained using an optical microscope (Nikon Eclipse E200, Nikon, Tokyo, Japan) and examined using NIS-Elements AR 3.2. (Nikon Instruments Europe B.V., Amsterdam, Netherlands). For each muscle fibre type, the fibre

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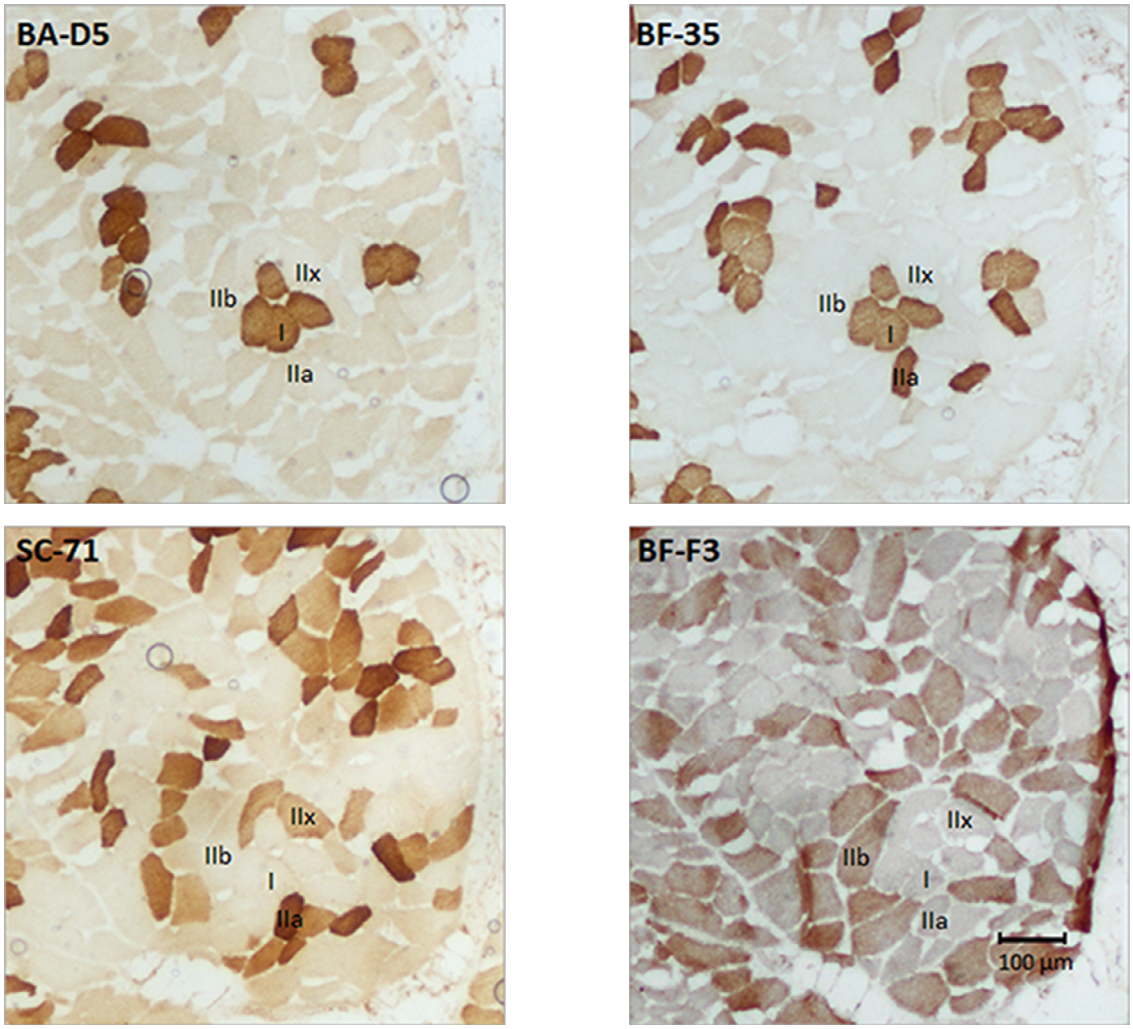

Figure 1. Serial sections of the porcine *longissimus lumborum* muscle stained with four monoclonal antibodies against specific myosin heavy chain (MyHC) isoforms: BA-D5 (MyHC I), BF-35 (MyHC I and IIa), SC-71 (MyHC 2a and 2x), and BF-F3 (MyHC IIb)

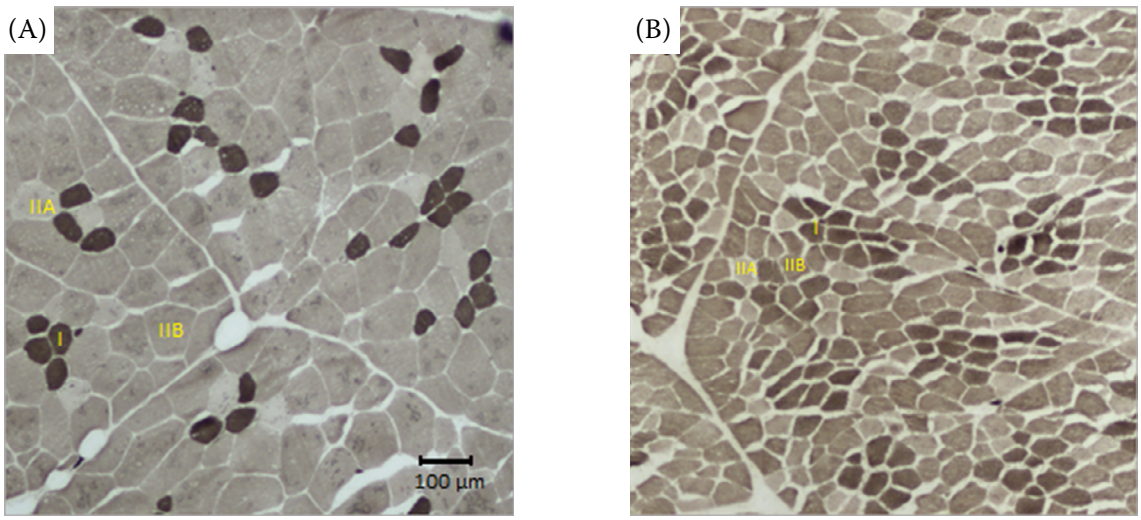

Figure 2. Porcine muscle fibres of *longissimus lumborum* (A) and *psoas major* (B) muscles stained by ATPase method after acid (pH 4.6) preincubation

https://doi.org/10.17221/23/2020-CJAS

cross-sectional area (CSA; μm^2), and fibre number/area proportion (percentage of individual fibre types in the total fibre number/relative area of fibres) were determined. Sample sections were also cut and stained with haematoxylin & eosin to measure CSAs for the estimation of these parameters for samples stained using the IHC method. The CSAs of fibres identified using the ATPase method were measured using slides stained in alkaline preincubation. Serial muscle slides were cut from the same block of the muscle sample, but the fields for analysis were chosen according the quality of samples and approximately 200 fibres were measured per sample.

Statistical analysis

The experimental data were analysed using SAS v9.4 statistical software (SAS Institute, Cary, NC, USA). Two-way analysis of variance (ANOVA) with fixed effects of muscle and staining method was used for evaluation of the muscle fibre composition. One-way ANOVA with the fixed effect of muscle was used for analysing the chemical composition of muscles. Results are presented as least squares means (LSMeans) ± standard error of the mean (SEM). Pearson correlation coefficients were calculated to evaluate the association between muscle fibre characteristics and the various meat quality traits. Differences are considered statistically significant at P-value < 0.05.

RESULTS

The results of the muscle fibre composition of LL and PM muscles stained using different methods are presented in Table 1, and the correlation coefficients between muscle fibre types and meat quality parameters for the LL muscle can be found in Table 2. The results of the chemical composition for LL and PM muscles are presented in Table 3, and their correlations with muscle fibre characteristics are shown in Table 4.

Interactions between the effects of muscle type and staining methods were calculated using the statistical model but no significant interactions were found for any fibre traits, and thus the main effects were interpreted separately. The PM muscle had

Table 1. Muscle fibre composition of porcine *longissimus lumborum* and *psoas major* muscles stained by immunohistochemical and ATPase method

Parameter	LL		PM		SEM	P-value	
	IHC	ATP	IHC	ATP		staining	muscle
Cross-sectional area (μm^2)							
All fibres	5 224	4 983	2 291	2 241	172.6	0.461	< 0.001
Type I	2 638	2 936	1 661	1 688	95.2	0.296	< 0.001
Type IIa	2 375	2 457	1 573	1 559	80.1	0.805	< 0.001
Type IIx	5 724	–	2 428	–	294.0	–	< 0.001
Type IIb/IIB	6 318	5 641	3 003	2 602	203.0	0.032	< 0.001
Fibre number proportion (%)							
Type I	11.0	10.9	20.1	17.5	0.68	0.227	< 0.001
Type IIa	15.0	10.7	22.5	21.3	0.66	0.003	< 0.001
Type IIx	19.6	–	21.4	–	0.82	–	0.281
Type IIb/IIB	54.4	78.4	36.1	61.3	1.69	< 0.001	< 0.001
Fibre area proportion (%)							
Type I	5.5	6.5	15.1	13.8	0.66	0.901	< 0.001
Type IIa	7.1	5.3	15.4	15.1	0.60	0.157	< 0.001
Type IIx	21.8	–	22.5	–	1.00	–	0.710
Type IIb/IIB	65.6	88.3	47.0	71.1	1.69	< 0.001	< 0.001

ATP = adenosine triphosphatase staining; IHC = immunohistochemical staining; LL = *longissimus lumborum*; PM = *psoas major*; SEM = standard error of the mean

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Table 2. Correlation coefficients between muscle fibre characteristics in the *longissimus lumborum* muscle and fresh meat quality parameters of Large White-Landrace crossbred commercial male pigs (live weight 105 ± 7.6 kg)

	LL area		pH_{45}		Drip loss		L*		a*		b*		WBSF raw		WBSF cooked	
Mean	5 138 mm^2		6.59		4.96%		52.45		–0.52		9.18		39.30 N		29.79 N	
	IHC	ATP	IHC	ATP	IHC	ATP	IHC	ATP	IHC	ATP	IHC	ATP	IHC	ATP	IHC	ATP
Cross-sectional area (μm^2)																
All fibres	–0.04	0.00	0.22	0.17	–0.03	–0.13	0.25	–0.06	–0.40*	–0.18	0.03	–0.10	–0.22	–0.11	–0.13	0.05
Type I	–0.16	–0.26	0.03	0.15	–0.04	–0.15	0.20	0.04	–0.39$^+$	–0.35$^+$	0.26	0.19	–0.16	0.05	–0.05	0.04
Type IIa	–0.01	–0.21	0.32	0.05	–0.12	–0.32	0.07	–0.07	–0.39$^+$	–0.20	–0.17	–0.08	–0.28	0.12	–0.07	0.29
Type IIx	–0.06	–	0.36$^+$	–	0.05	–	0.23	–	–0.49*	–	–0.05	–	–0.14	–	–0.29	–
Type IIb/IIB	0.06	0.05	0.17	0.15	–0.04	–0.13	0.24	–0.08	–0.26	–0.13	0.03	–0.14	–0.22	–0.11	–0.11	0.07
Fibre number proportion (%)																
Type I	–0.40*	–0.32	0.29	0.22	–0.46*	–0.38$^+$	–0.12	–0.30	–0.17	0.09	–0.34$^+$	–0.22	0.25	0.27	0.28	0.32
Type IIa	0.45*	0.44*	–0.07	–0.23	0.25	0.02	0.01	0.04	0.31	0.15	0.20	–0.04	–0.09	0.03	–0.28	0.10
Type IIx	0.45*	–	–0.36$^+$	–	0.34$^+$	–	0.30	–	0.06	–	0.22	–	–0.38$^+$	–	–0.22	–
Type IIb/IIB	–0.55**	–0.07	0.21	–0.01	–0.24	0.29	–0.21	0.21	–0.21	–0.19	–0.17	0.21	0.28	–0.24	0.27	–0.34
Fibre area proportion (%)																
Type I	–0.46*	–0.44*	0.15	0.19	–0.41*	–0.37$^+$	–0.07	–0.16	–0.26	–0.07	–0.07	0.03	0.22	0.37$^+$	0.25	0.25
Type IIa	0.26	0.20	0.10	–0.23	0.07	–0.17	–0.12	0.01	0.30	0.10	0.12	–0.05	–0.03	0.18	–0.10	0.22
Type IIx	0.37$^+$	–	–0.18	–	0.33	–	0.22	–	0.01	–	0.13	–	–0.25	–	–0.31	–
Type IIb/IIB	–0.33	0.27	0.10	–0.02	–0.24	0.44*	–0.15	0.14	–0.05	0.00	–0.15	0.00	0.19	–0.46*	0.27	–0.38$^+$

ATP = adenosine triphosphatase staining; IHC = immunohistochemical staining; LL = *longissimus lumborum*; pH_{45} = pH at 45 minutes *post-mortem*; WBSF = Warner-Bratzler shear force

$^+$0.1; *0.05; **0.01

smaller CSA for all fibre types compared with the LL muscle ($P < 0.001$). The type IIb fibres had the largest CSA in both muscles, followed by IIx, I and IIa fibres (Table 1). The fibre type proportion also differed between muscles ($P < 0.001$); the LL muscle had a lower proportion of type I and IIa fibres and a higher proportion of type IIb fibres than the PM. However, the proportion of IIx fibres did not differ between the two muscles. Staining method affected the determination of the fibre parameters of type IIb/IIB ($P < 0.001$). In terms of the fibre type proportion, there were also differences in type IIa fibres between the staining methods ($P < 0.003$; Table 1).

The correlations between the proportion of type I fibres and drip loss were negative (Table 2). Furthermore, moderate negative correlations were observed between the type I fibre relative area and the LL eye muscle area. Positive correlation coefficients were observed for the proportion of type IIa and IIx and LL eye muscle area, but a higher proportion of type IIb (IHC method) was negatively correlated with the LL eye muscle area. The fibre area proportion of type IIB identified using the ATPase method was positively correlated with drip loss, and negatively with the WBSF of raw meat. The CSAs of all fibre types identified using the IHC method were negatively correlated with a* colour values of the LL muscle surface (Table 2).

The PM muscle had higher moisture ($P < 0.001$), crude protein ($P < 0.05$), and IMF values ($P < 0.001$) than the LL muscle (Table 3). Moisture and ash contents were negatively correlated with the CSA of all

Table 3. Chemical composition of *longissimus lumborum* and *psoas major* muscles from Large White-Landrace crossbred commercial male pigs (live weight 105 ± 7.6 kg) on an as-is basis

Parameter (%)	LL	PM	SEM	*P*-value
Moisture	73.7	75.0	0.18	< 0.001
Crude protein	23.4	24.0	0.18	0.047
Intramuscular fat	1.6	2.0	0.06	< 0.001
Ash	1.2	1.3	0.02	0.591

LL = *longissimus lumborum*; PM = *psoas major;* SEM = standard error of the mean

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Table 4. Correlation coefficients between muscle fibre characteristics in *longissimus lumborum* and *psoas major* muscles and their chemical composition in Large White-Landrace crossbred commercial male pigs (live weight 105 ± 7.6 kg)

Trait	LL								PM							
	moisture		crude protein		fat		ash		moisture		crude protein		fat		ash	
	IHC	ATP	IHC	ATP	IHC	ATP	IHC	ATP	IHC	ATP	IHC	ATP	IHC	ATP	IHC	ATP
Cross-sectional area (μm^2)																
All fibres	–0.42*	–0.43*	0.08	–0.02	0.01	0.02	–0.58**	–0.55**	–0.04	0.03	–0.01	0.19	–0.10	–0.27	–0.03	0.00
Type I	–0.32	–0.45*	0.03	–0.04	0.01	–0.05	–0.36$^+$	–0.49*	–0.06	–0.01	0.13	0.18	–0.01	–0.10	0.04	0.07
Type IIa	0.10	–0.42*	–0.04	–0.13	–0.13	–0.02	–0.07	–0.44*	0.02	–0.17	0.06	0.21	–0.14	–0.18	–0.01	0.02
Type IIx	–0.08	–	0.24	–	0.25	–	–0.23	–	–0.01	–	–0.04	–	–0.16	–	–0.11	–
Type IIb/IIB	–0.43*	–0.38$^+$	0.02	–0.05	–0.10	0.04	–0.62**	–0.47*	–0.11	0.07	–0.02	0.21	–0.10	–0.27	0.05	–0.03
Fibre number proportion (%)																
Type I	–0.12	–0.25	0.24	0.07	0.04	0.14	–0.10	–0.15	0.01	–0.08	0.20	0.34$^+$	0.00	0.23	0.17	0.09
Type IIa	0.21	0.30	–0.34	–0.36	–0.12	0.00	–0.03	0.45*	–0.20	–0.38$^+$	0.27	0.16	0.04	0.21	–0.18	–0.03
Type IIx	0.31	–	0.01	–	0.01	–	0.21	–	0.03	–	–0.30	–	–0.03	–	0.44*	–
Type IIb/IIB	–0.38$^+$	–0.02	0.11	0.21	0.07	–0.11	–0.11	–0.21	0.10	0.23	–0.13	–0.32	–0.01	–0.26	–0.30	–0.06
Fibre area proportion (%)																
Type I	–0.14	–0.35$^+$	0.23	0.04	0.00	0.08	–0.02	–0.19	0.02	–0.10	0.25	0.29	0.05	0.28	0.23	0.13
Type IIa	0.35$^+$	0.25	–0.41$^+$	–0.31	–0.16	–0.09	0.10	0.39$^+$	–0.11	–0.46*	0.34$^+$	0.21	–0.04	0.19	–0.15	0.03
Type IIx	0.40*	–	0.13	–	0.15	–	0.24	–	0.05	–	–0.34$^+$	–	–0.09	–	0.35$^+$	–
Type IIb/IIB	–0.47*	0.15	–0.06	0.20	–0.08	–0.02	–0.26	–0.07	0.00	0.29	–0.12	–0.29	0.04	–0.27	–0.32	–0.10

ATP = adenosine triphosphatase staining; IHC = immunohistochemical staining; LL = *longissimus lumborum;* PM = *psoas major*
$^+$0.1; *0.05; **0.01

fibres for both ATPase and IHC staining methods (Table 4). No significant correlations were found for fibre CSA and chemical composition of PM muscle. For the LL muscle, moisture content was negatively correlated with the fibre area proportion of type IIb, however this was not observed for ATPase staining. A positive correlation was found for moisture and fibre area proportion of type IIx. Ash content was positively correlated with the fibre number proportion of type IIa stained by ATPase method in LL muscle, and with the fibre number proportion of type IIx in PM muscle. For the PM muscle, negative correlations were observed for moisture content and the fibre area proportion of type IIa in the ATPase assay ($P < 0.05$), which in case of IHC staining was not significant (Table 4).

DISCUSSION

Muscle fibre characteristics are influenced by muscle function and location, and in general, the deep muscles involved in maintaining posture are more oxidative and contain more type I fibres than the superficial muscles involved in fast movements (Joo et al. 2013). Similarly to the results from the present study, Chang et al. (2003) observed a higher proportion of type IIa and IIx fibres, and a lower proportion of type IIb fibres in the PM than in the LL muscle of selected pig breeds (Table 1). However, Chang et al. (2003) did not observe a higher proportion of type I fibres in the PM muscle of Large White pigs, as was the case in the present study involving Large White crossbreds. Nonetheless, the present study is in accordance with previous results reported by Lefaucheur and Vigneron (1986) and Kim et al. (2017).

There are not many studies focused on IHC staining of muscle fibres in the PM muscle of pigs. The results of classical histochemical staining (ATPase, SDH, NADH-TR) confirm that there is a higher proportion of slow oxidative (I) and fast oxido-glycolytic (IIA) fibres in the PM muscle compared with the LL muscle of pigs (Velotto et al. 2012; Losel et al. 2013; Hwang et al. 2018). In addition, Velotto et al. (2012) observed higher CSAs of all three fibre types in the LL than in PM muscle,

https://doi.org/10.17221/23/2020-CJAS

which is consistent with the results of the present study (Table 1).

Differences in the proportion of type IIb/IIB fibres between the two staining methods were expected, because the ATPase method does not discriminate between type IIx and IIb. Therefore, IHC staining is more appropriate for the detection and evaluation of fibre types in pigs, as all three fast MyHC isoforms are expressed in porcine muscle (Lefaucheur 2010; Fazarinc et al. 2013). In comparison with other large livestock animals such as cattle, porcine muscles have a high proportion of IIB fibres identified using ATPase staining, and in the case of the LL muscle, the relative area of IIB fibres is reported to be between 69% to 90%, depending on pig breed and age (Ruusunen and Puolanne 2004; Ryu et al. 2008). However, when the relative area of IIb fibres in porcine LL muscles is evaluated using IHC staining, the relative area is reported to be 26% to 65% (which corresponds to the results of the present study) depending on the breed and the fibre classification system used [if hybrid fibres are included or not; Chang et al. (2003); Fazarinc et al. (2013); Kim et al. (2013)]. Thus, IIx fibres may actually contribute up to 50% of the volume of fibres IIB evaluated using ATPase staining. Furthermore, IHC staining methods also provide more accurate results in *post-mortem* samples with reduced ATPase enzyme activity, which is crucial for some classification techniques (Pette and Staron 2000). The staining pattern of the myosin ATPase is ambiguous due to its pH sensibility, and can lead to the misclassification of fibre types (Fazarinc et al. 2013). It is therefore expected that the differences reported for the relative area of fibres IIa between the staining methods used in the present study may be due to the fact that hybrid fibres were included in their estimation when the ATPase method was used. No differences were observed for fibre type I between staining methods (Table 1) confirming the statement that slow fibre types correspond well between staining techniques (Klont et al. 1998; Behan et al. 2002).

Differences in meat quality traits are related to differences in the composition of muscle fibres in the skeletal muscles of animals (Lee et al. 2010; Lefaucheur 2010; Joo et al. 2013). In the present study, the effect of muscle fibre composition in only the LL muscle stained by two different methods on carcass and meat quality traits was evaluated (Table 2). Increasing proportions of fibres IIb/IIB are usually associated with detrimental effects on meat quality in pigs, such as lower water-holding capacity, rapid rate of pH decline and paler surface colour, while type I fibres have the opposite, more favourable properties (Choe et al. 2008; Lefaucheur 2010; Joo et al. 2013; Su et al. 2013). Muscle fibres are dynamic structures that exhibit high plasticity and can shift from one to another type in the following pathway: I ↔ IIa ↔ IIx ↔ IIb, and thus the biological characteristics of muscle fibre types differ as well. For example, type I is associated with high contents of myoglobin, lipids and vascularization, as well as low contraction speed and threshold, whereas type IIb displays the opposite for these properties (Pette and Staron 2000; Lefaucheur 2010; Schiaffino and Reggiani 2011; Joo et al. 2013; Listrat et al. 2016). Higher proportions of type I fibres are also associated with smaller LL eye muscle areas (Lefaucheur 2010; Joo et al. 2013; Kim et al. 2013), which is also confirmed by the present study. Despite the presence of IIb *MyHC* gene in all mammals, the type IIb MyHC isoform is not expressed in all large mammals (Lefaucheur 2010; Listrat et al. 2016). Nevertheless, a strong expression of IIb MyHC is observed in pig breeds selected for leanness and high growth performance (Listrat et al. 2016), but the high abundance of IIb fibres in this classification could be expected to be associated with problematic meat parameters of pork. Unexpectedly, the present study shows that these parameters could be associated with muscle fibres IIx. The trend of the correlations shows that a higher content of IIx fibres could be associated with a higher LL eye muscle area, lower pH, and higher drip loss, whereas higher proportions of type IIb were negatively correlated with the LL eye muscle area, and they were not related with meat quality. This could be related to many factors, such as breed, age of animals or the selected staining technique. Moreover, differences in sampling time and location can affect the results because intramuscular variability in fibre type composition has been described (Realini et al. 2013). Water-holding capacity is a major quality parameter for fresh pork, which is related to the extent of protein denaturation (Su et al. 2013). Glycolytic metabolism predominates in muscle fibres of type IIB, which contributes to a rapid decrease in pH values in the early *post-mortem* stage. This is a particular problem in pork meat which contains a higher proportion of type IIB muscle fibres compared to beef or lamb meat. In accordance with the present results, other studies show a posi-

https://doi.org/10.17221/23/2020-CJAS

tive effect of higher type I fibre content on reducing drip losses of meat (Kim et al. 2013; Lee et al. 2016). However, Chang et al. (2003) did not observe any significant correlations for drip loss and proportion of MyHC I, IIx and IIb isoforms in the *longissimus dorsi* muscle and in the PM muscle, the conclusions regarding their relationship differed according to the breed. The correlation between meat drip loss and MyHC 2b was negative in Berkshire pigs and positive in Tamworth pigs, but in MyHC 2x, this correlation coefficient was positive in Berkshire and negative in Tamworth pigs. However, it is expected that the low sample number of pigs would have an influence on the correlations, as was observed in the present study, and in that of Chang et al. (2003). Kim et al. (2013) focused their study on the muscle fibre characteristics and meat quality traits in pork with diverse meat abnormalities (dark, firm and dry – DFD; pale, soft and exudative – PSE; reddish pink, firm and non-exudative – RFN; reddish pink, soft and exudative – RSE). They also observed significant correlations between muscle fibre parameters and meat quality, but the comparison with the present study is limited because they evaluated six types of muscle fibres. In addition, Kim et al. (2013) examined the hybrid types IIax and IIxb, and revealed significant correlations between these hybrid types and meat quality parameters. Dissimilar results obtained in the present study could be partially explained by the existence of these hybrid fibres, which are included in one of the three fast fibre types according to the predominance of the MyHC isoform. For example, Kim et al. (2013) reported negative correlations between drip loss and relative area of IIax fibres, and also negative correlations for sarcoplasmic protein solubility and relative area of type IIxb, which affects meat tenderness. Thus, the inclusion of hybrid fibres to one of the pure fibre types can significantly affect the results for the relationships between muscle fibre characteristics and meat quality parameters, which is also documented in Table 2. In many cases like fibre composition and drip loss or WBSF, differences in the results of staining methods used in studies can be observed. With ATPase staining, all fibre types – IIx, IIb and IIxb (IIaxb) – are included in the classification of IIB fibre type, while unexpected results for IIx and IIb fibres in IHC method may also be due to the misclassification of hybrid fibres. The discrepancies between the results of different studies can also be explained by the existence of other protein isoforms, such as myosin light chain, troponin or tropomyosin isoforms (Choi and Kim 2009). For example, Oe et al. (2007) reported that tropomyosin isoform composition can affect *post-mortem* changes in meat and thus ultimately the meat quality. Nevertheless, the effect of these protein isoforms on meat quality traits is not fully established (Choi and Kim 2009). No significant correlations were found between fibre CSA and meat quality traits in the present study, except for a*, which was negatively correlated with fibre CSA when the IHC method was used. Meat with higher concentrations of myoglobin is redder and has higher levels of "red fibres", namely type I and IIA (Listrat et al. 2016). These fibres, compared to fibres of type IIB, also contain more blood capillaries which also cause a darker meat colour (Su et al. 2013). Capillary density also depends on the mean CSA of muscle fibres, which is often modulated in response to changes in physical demand or to the metabolic environment (Olfert et al. 2016). Negative correlations for a* and fibre CSA were observed only with IHC fibre typing and not with the ATPase fibre typing in the present study, which could be due to the measurement of CSAs in different images.

Differences in the chemical composition of LL and PM muscles between the muscles were observed in the present study (Table 3), and some significant correlations were revealed between these chemical parameters and muscle fibre characteristics (Table 4). Many correlations were found for muscle fibre parameters and the moisture and ash content of LL muscle, with fibre CSA being negatively correlated with these parameters. These correlations were not observed in the PM muscle, although the moisture and ash content was higher, and CSA smaller, in the PM muscle than in the LL muscle. Kim et al. (2013) determined the correlations between moisture content and muscle fibre parameters in pork, but they did not observe any significant correlations for moisture content and CSA. They however found positive correlations for moisture content and relative area of IIb fibres. In the present study, this correlation was negative, but Kim et al.'s (2013) study examined six muscle fibre types. Negative correlations between muscle moisture content and the proportion of IIb fibres could be linked with the WHC of meat. It is commonly known that muscles containing higher proportions of IIb/IIB fibres have lower WHC, which in turn is also associated with higher CSA

https://doi.org/10.17221/23/2020-CJAS

of fibres (Lefaucheur 2010; Joo et al. 2013; Kim et al. 2013). However, in the PM muscle, this negative correlation was observed for IIA fibres determined using the ATPase method. Explanation for this opposing result between the two muscles may be attributed to a broad range of intrinsic biochemical and biophysical characteristics that could exist in each fibre type, introducing variations to a range of meat characteristics in different muscles, as well as in different pig breeds (Chang et al. 2003). Several studies show that chemical and biochemical components differ between muscles with diverse fibre type compositions, affecting meat quality traits. Hwang et al. (2018) observed that the LL and PM muscles differ in their nucleotide compounds, including inosine monophosphate, adenosine monophosphate and hypoxanthine. These compounds can even influence some sensory parameters of meat (Hwang et al. 2018), and their different concentrations may form part of the determination of the ash content of meat. The ash content is a measure of the total amount of minerals present in meat; however, the main compounds represented by this fraction are K, P, Mg, Na and Ca, and all of them are important for muscle fibre contraction. Soglia et al. (2015) observed lower moisture and fat contents coupled with higher amounts of protein and ash, and decreased calcium and sodium levels in normal compared to wooden breast of broilers, which is typical of the histopathological changes, such as an increase in degenerative and atrophic fibres associated with loss of cross striations, variability in fibre size, or degeneration and lysis of fibres. Another influencing factor could be the sarcomere length; Guzek et al. (2015) stated that the sarcomere length is positively correlated with intramuscular fat content and negatively correlated with protein content. It has been indicated that type IIB fibres are characterized by a shorter sarcomere length than type I fibres (Christensen et al. 2006).

Although the PM muscle had a significantly higher content of IMF, as shown in Table 3, as well as a higher content of red fibres I and IIa than the LL muscle (Table 1), no significant correlations between IMF content and fibre parameters were observed in the present study. Kim et al. (2013) reported positive correlations for IMF content and CSA of IIa and IIax fibres in the LL muscle. In agreement with the present study, Candek-Potokar et al. (1999) did not observe any significant correlations between IMF content and muscle fibre characteristics. The present results thus further support the conclusion that there is no universal relationship between muscle fibre composition and IMF content in meat (Lefaucheur 2010).

Unfortunately, meat quality traits could not be measured in the PM muscle in the present study, as the collection of the samples formed part of a larger project and the PM muscles were used for sensory evaluation. Nevertheless, the present study highlights the fact that using the IHC staining technique would better describe the relationships between the composition of muscle fibres and meat quality.

Future studies describing the histological composition of muscle fibres and meat quality should be focused on chemical and enzymatic parameters of fast muscle fibre types in pigs, which according to the results of the study, form a significant proportion of high-value muscles in pigs.

CONCLUSION

The effect of two staining methods on the determination of relationships between fibre type composition and pork meat quality was investigated in this study, revealing that IHC fibre typing is more appropriate than ATPase fibre typing for the evaluation of porcine muscles, and should be used to assess the relationships between fibre type composition and meat quality traits. The study also confirms a number of important conclusions, including that the LL and PM muscles exhibit different fibre type compositions, and that the relative area of type I fibres in the LL muscle has a positive effect on the reduction of drip loss and a negative effect on the LL eye muscle area.

Conflict of interest

The authors declare no conflict of interest.

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