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The metabolic properties of quadriceps femoris muscles of Lika pramenka sheep breed, fed with a supplement from button mushrooms (*Agaricus bisporus*)

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ABSTRACT

The objective of this study was to verify the presence of oxidative enzymes in the quadriceps femoris muscle of Lika Pramenka sheep and to evaluate the effect of supplement from button mushrooms, *Agaricus bisporus* (*A. bisporus*), on the histochemical and morphological characteristics of the muscles. Fourteen one-year-old Lika Pramenka sheep were randomly divided into two groups (control and experimental groups) comprising 7 animals each. The experiment was conducted over a period of 6 weeks of the winter feeding period. The control group of sheep was fed with a standard feed without antimicrobials or growth promoters. The experimental group was given the same feed but with an additional 1.5% of dry *A. bisporus.* During the experiment access to water and feed was *ad libitum*. Samples were tested for the size and type of muscle fibers, as well as for the presence of oxidative enzymes. The diameter of muscle fibers and histochemical reactions profiles were significantly greater in animals fed with the *A. bisporus* supplement. On the basis of the increased activity of oxidative enzymes, which resulted in pronounced cell metabolism and increased muscle mass, we conclude that *A. bisporus* displays growth promoter effects.

Key words: *Agaricus bisporus,* oxidative enzymes, Lika Pramenka sheep breed

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Introduction

Mushrooms are known to have active substances that display antifungal, anti-inflammatory, antiviral, antibacterial, hepatoprotective, antidiabetic, hypolipidemic, antithrombotic and hypotensive activities (WASSER, 2002; WASSER, 2010). The ratio of muscle fiber types determines muscle appearance, and physiological and bi**o**chemical characteristics. In general, muscle fibers are differentiated according to their contractile (slow or fast twitch) and metabolic properties (oxidative, glycolytic) (ČANDEK-POTOKAR et al., 1998). Muscle energy metabolism relies on fuels from both extra- and intra- muscular sources - mainly glucose, lactate, non-esterified fatty acids, triglycerides and glycogen. The amount of ATP generated from each of these energy sources and the selection of appropriate substrates for energy production depend, among other factors, on the metabolic type and contractile activity of the muscle tissue (HOCQUETTE et al., 1998; CHRIKI et al., 2013). It has been suggested that these metabolic fiber types are to be referred to as type I and type II respectively. Type I fibers correspond to 'red' muscle and probably depend on oxidative metabolism (Krebs cycle) for their energy, whereas the type II fibers, corresponding to 'white' muscle, probably obtain their energy mainly from the anaerobic glycolytic pathway. In most muscles, fibers have also been observed with intermediate activity between the strong and the weak fibers, for a particular enzyme reaction. More recently enzymatic reactions are being used to visualize different types of muscle fibers according to their contractile, metabolic and physiological properties. In respect of these properties we can distinguish several types. Slow twitch oxidative (STO) fibers are small in diameter and show low levels of glycolytic enzymes, a moderate to high degree of aerobic activity, a positive and negative reaction to nicotinamide adenosine dinucleotíde tetrazolium reductase (NADH-TR) and m-ATPase after acid and alkaline preincubation, respectfully. Fast twitch oxidative glycolytic (FOG) fibers exhibit an intermediate diameter, a moderate to high degree of aerobic activity, coupled with high concentrations of oxidative and cytochrome enzymes. Reactions to (NADH-TR) and m-ATPase after acid and alkaline are respectfully negative and moderate. The third type of fibers are fast twitch glycolytic (FG), which have a large diameter, and high levels of glycogen, while the activity of oxidative and cytochrome enzymesis is low. They also exhibit a weak reaction to NADH-TR and a strong reaction to m-ATPase after alkaline, and strong after acidic preincubations (DE MACEDO et al., 2000).

An increase in meat production may be achieved by increasing muscle mass, which in turn can be accomplished through the growth in size or number of muscle fibers. The positive effects of button mushrooms, *Agaricus bisporus* (*A. bisporus*), on various aspects of animal physiology are widely known (CHANG and MILES, 2004; WUD et al., 2007; CHANG and WASSER, 2012; REIS et al., 2012). Accordingly, the objective of this research was to evaluate the muscle histochemistry and growth characteristics of sheep treated

with a dry supplement of *A. bisporus* and determine any growth promoter effect. The investigation of muscle fiber characteristics could be of practical importance to provide scientists, breeders, and industry with a better understanding of muscle fiber involvement in determining muscle growth and final meat quality traits.

Materials and methods

The mushrooms. Commercial dried mushroom powder (*A. bisporus)* was obtained from a mushroom producer, and 100 g of powder contained 59.44% of protein, 31.51% of carbohydrates and 6.32% of ash (GEA-COM d.o.o., Croatia).

The sheep. Fourteen one-year-old female Lika Pramenka sheep were obtained from a commercial rearing farm (The Živković Farm, Kvarte, Perušić, Croatia). All procedures used in this research were in compliance with the European guidelines for the care and use of animals in research (Directive 2010/63/EU).

Study design and procedures. Fourteen one-year-old female Lika Pramenka sheep were randomly divided into two groups (control group and experimental group) comprised of 7 animals each. The experiment was conducted over a period of 6 weeks in the winter feeding period. The control group was fed with a standard diet, while the experimental group was given a standard diet supplemented with an additional 1.5% dry *A. bisporus.* The standard diet was based on pasture and concentrate, containing corn (66.3%), soybean meal (18.7%), bran (6%) and alfalfa meal (4%). During the experiment access to water and mineral salt blocks was always available. Tissue samples were obtained from each animal after technological slaughter, from the bellies of the m. quadriceps femoris.

Histochemical and morphological analysis. The collected samples were immediately frozen in liquid nitrogen (-196 °C) and stored at -80 °C until histochemical and morphological analyses were conducted. Transverse serial sections were cut in cryostat at -20 °C and transferred to glass cover slips. For morphological evaluation of the muscles, sections were stained with hematoxylin and eosin (HE), according to the protocol provided by LILLIE (1954). To evaluate the oxidative activity of the muscle fibers, the sections were stained histochemically for succinate dehydrogenase (SDH), lactate dehydrogenase (LDH), and NADPH-diaphorases, following CHAYEN and BITENSKY's (1991) protocol. Stained sections were examined microscopically and photographed. Fiber diameter was determined according to CARPENTER et al. (1996). Fiber diameter and enzymatic reaction intensity profiles were determined using the image-analyzing system Image J. The diameters of each fiber type from each animal were then averaged to obtain the mean muscle diameter for that fiber type for each animal. For calculating the percentage of oxidative and glycolytic fibers, sections were analyzed by relating the number of counted fibers of each type to the total fiber number counted. Muscle fiber proportions and sizes were compared using Student's *t* test.

Results

Histochemical and morphological evaluation. Morphological analysis of the muscle fibers showed normal multinucleated fibers, with peripheral nuclei separated by the endomysium and grouped in fascicles by the connective tissue of the perimysium. Histochemical analysis revealed two fiber types: 1) oxidative fibers with intense or moderate enzyme activity, with reaction product clusters distributed in the subsarcolemmal or intermyofibrillar regions, and 2) glycolytic fibers with weak enzyme activity and small clusters of reaction product in the fiber sarcoplasm. Fiber frequency was similar in both groups of animals (Table 1). At the end of the experiment, the diameter of both fiber types, oxidative and glycolytic, was significantly higher in the experimental group of animals (Table 2). Histochemical reaction profiles were similar in both groups. However, a higher intensity of all reactions was noticed in the quadriceps femoris muscle of animals fed with the *A. bisporus* supplement. The activities of the oxidative enzymes studied are presented in Table 3. SDH had a moderate reaction in the quadriceps femoris muscle of the experimental group of animals, and a slightly weaker reaction in the control group of animals (Fig. 1A, B, C, D). Staining intensity for LDH was strong in the experimental group and moderate in the control group of animals (Fig. 2A, B, C, D). The activities of NADPH-diaphorases are given in Fig. 3A, B, C, and D. NADPH-diaphorases gave a moderate to strong reaction in the quadriceps femoris muscle in both groups of animals.

Table 1. Number of oxidative and glycolitic fibers in quadriceps femoris of the Lika Pramenka, fed with the *A. bisporus* supplement (experimental group) and with the basal diet (control group)

	Number* of oxidative and glycolitic fibers in quadriceps femoris of the Lika Pramenka	
Type of muscle fibers	Control group	Experimental group
Oxidative	47 ± 2.64	53.5 ± 8.02
Glycolitic	53 ± 5.29	46.5 ± 8.01
Total	100 (100%)	100 (100%)

* numbers are representative Means ± SD of seven animals per group

Table 2. Diameter of the oxidative and glycolitic fibers (μm) of quadriceps femoris of the Lika Pramenka, fed with the *A. bisporus* supplement (experimental group) and with the basal diet (control group)

	Diameter* of oxidative and glycolitic fibers in quadriceps femoris of the Lika Pramenka		
Type of muscle fibers	Control group	Experimental group	P-value
Oxidative	35.31 ± 5.15	45.96 ± 5.15	0.0022
Glycolitic	42.07 ± 5.32	54.43 ± 6.70	0.0026

* numbers are representative Means ± SD of seven animals per group

Table 3. Activity of oxidative enzymes in quadriceps femoris of Lika Pramenka, fed with the *A. bisporus* supplement (experimental group) and with the basal diet (control group)

	Activity* of oxidative enzymes in quadriceps femoris of Lika Pramenka	
Oxidative enzymes	Control group	Experimental group
Succinate dehydrogenase (SDH)	+++	++
lLactate dehydrogenase (LDH)	++++	+++
NADPH-diaphorases	+++	++

* numbers are representative Means ± SD of seven animals per group. Staining intensities are valued as: 0 no reaction visible, + very weak, ++ weak, +++ moderate, ++++ strong.

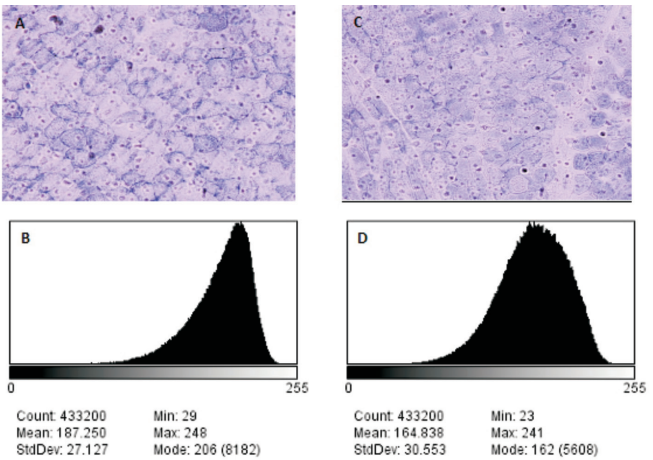

Fig. 1. SDH activity (A, C) and measured staining intensity (B, D) in the quadriceps femoris muscle of Lika Pramenka sheep fed with the basal diet (A, B) and with the *A. bisporus* supplement (C, D). The numbers represent the pixel density of the corresponding image

* Numbers are representative Means ± SD of seven animals per group

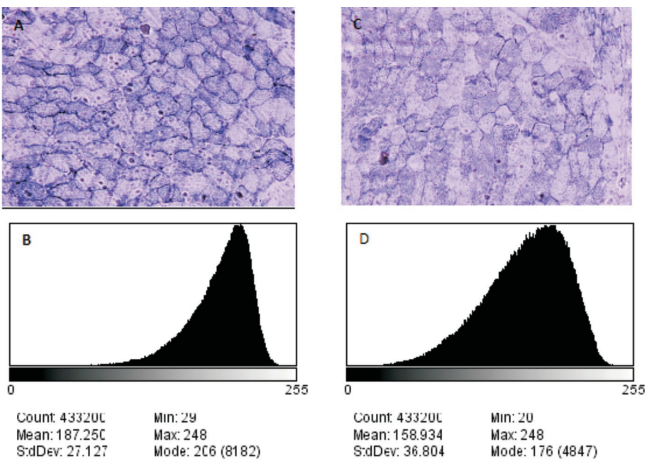

Fig. 2. LDH activity (A, C) and measured staining intensity (B, D) in the quadriceps femoris muscle of Lika Pramenka fed with the basal diet (A, B) and with the *A. bisporus* supplement (C, D). The numbers represent the pixel density of the corresponding image.

* Numbers are representative Means ± SD of seven animals per group

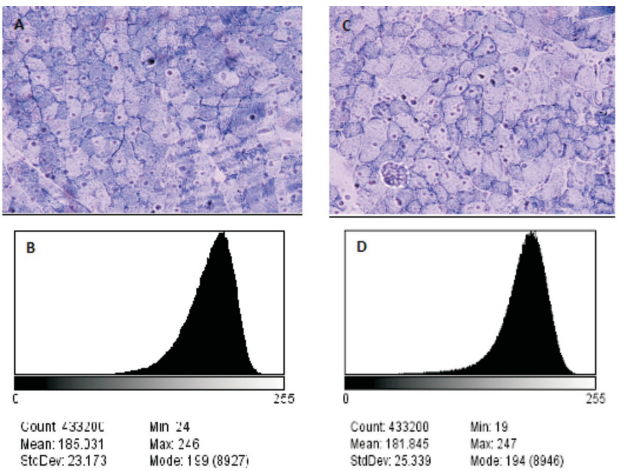

Fig. 3. NADPH activity (A, C) and measured staining intensity (B, D) in the quadriceps femoris muscle of Lika Pramenka fed with the basal diet (A, B) and with the *A. bisporus* supplement (C, D). The numbers represent the pixel density of the corresponding image.

Discussion

Mushrooms contain numerous biologically active substances (such as glucans, mannans, lentinans, griffolyns, schizophyllans, scleroglucans) with certain antibacterial, antiviral, antitumoral and immunostimulatory effects (WASSER, 2002; DALLA-SANTA et al., 2010; WASSER, 2010). Reduced cholesterol concentration, regulation of blood pressure, blood sugar and digestion, enhancement of the respiratory system, and stimulation or depression of the central nervous system (JEONG et al., 2010) are some of their most important outcomes. Additionally, mushrooms are a source of numerous antioxidants, vitamins A, C, D and E, minerals such as phosphorus, potassium and iron, and also have a noticeable nutritional activity. *A. bisporus* is one of the most frequently cultivated edible mushrooms in the world. It contains 5.52% of dry substance with 59.44% of proteins, 31.51% of carbohydrates and 6.32% of ash (NOVAK, 1997). Good nutritive properties with low fat and high protein and carbohydrate content, make it a very acceptable food, not only for humans, but also for domestic animals intended for human consumption. It has been shown that β-glucans, isolated from *A. bisporus*, beside their antitumor effect, also act as immunostimulants to the systemic and local (gut) immunity of some species of farm animals (BROWN and GORDON, 2003; SHEN et al., 2007; BARBISON et al., 2010, MRŠIĆ et al., 2011). Previous studies have demonstrated that supplementation of animal feed with *A. bisporus* improves feed conversion and animal growth, but the exact mechanism of growth promotion is still not known. Moreover, on the basis of the percentages of water, protein, fat and ash in the meat of broilers fed with *A. bisporus* supplement, MRŠIĆ et al. (2013) concluded that the meat had a significantly lower fat content. The composition of carcasses varies, not only according to species, but also according to breed, age, and sex and feed type.

Fiber type distribution within muscles is of significance when studying fiber type composition in relation to meat quality (CARANI et al., 2006). A wide range of strategies are available for inducing changes in different meat constituents. These include: genetic selection, growth-promoting and nutrient partitioning agents, immunization of animals against target circulation hormones or releasing factors, gene manipulating techniques, as well as nutrition and feeding management (JIMÉNEZ-COLMENERO et al., 2001). The present study evaluated the effect of dry *A. bisporus* supplement in feed on muscle fiber type frequency, fiber size and histochemical reaction profiles. The histochemical assay for SDH and NADH was used to distinguish between oxidative and non-oxidative, more accurately "less" oxidative fibers. Given the fact that fibers with high oxidative capacity generate ATP via oxidative phosphorylation in the mitochondria, it is to be expected that muscle cells which contain more mitochondria will have higher oxidative capacity. The SDH enzyme is located in the inner membrane of the mitochondria, bound to the cristae. SDH is responsible for oxidizing succinate to fumarate in the citric acid cycle. As this

reaction proceeds, the succinate is oxidized, and the reduced form of NADH is produced. The more mitochondria (and therefore SDH) a fiber contains, the staining will be of higher intensity. Oxidative fibers have a relatively dense, purple speckled appearance, while non-oxidative fibers have only scattered purple speckles. Therefore this histochemical assay reflects the relative oxidative potential of muscle fibers (SOUKUP et al., 1979). While the histochemical demonstration of SDH activity indicates the metabolic activity of the fibers, it does not establish the speed of contraction of the ovine fibers. According to the literature data, ovine muscles contain three different muscle fiber types, based on histochemically staining for myosin ATPase: type SO (slow-contracting with oxidative metabolism) or I fibers, FOG (fast contracting - with glycolytic-oxidative metabolism) or IIA, and FG (fast-contracting with glycolytic metabolism) or II B fibers (VELOTTO et al., 2010). The percentage and distribution of different types of fibers, classified by means of the m-ATPase technique, are genetically determined in the semitendinosus, triceps brachii and abdominal cutaneous muscles. However, the percentage of m-ATPase fiber types changes after birth in the quadriceps and longissimus muscle (ASHMORE et al., 1972; SUZUKI and TAMATE, 1988; WHITE et al., 1978; MOODY et al., 1980). Future research will be aimed at evaluating the possible effect of *Agaricus bisporus* supplement on the speed of contraction of ovine myofibers.

Increased activity of oxidative enzymes, SDH, LDH, NADH, NADPH, in the quadriceps femoris muscle of animals fed with *A. bisporus* supplement compared to the control group of animals, indicated an increased cellular metabolism. Fiber type frequencies (oxidative and glycolitic fibers) were similar in both groups of animals, which is in concordance with the results of FAHEY et al. (2005) and HEGARTY and ALLEN (1978), who stated that the majority of muscle differentiation and fiber formation takes place in utero, at approximately day 85 of gestation, and cannot be manipulated in a wide range post-natally. On the other hand, nutrition and feeding management could affect muscle growth. During the experiment, the experimental group of animals showed a significant increase in the fiber cross-sectional diameter of both types of muscle fiber.

Conclusion

The results of this research indicate the growth promotion effect of *A. bisporus*, on the basis of the increased activity of oxidative enzymes, which resulted in pronounced cell metabolism. It is noteworthy that this study provides useful information to allow the design of future experiments aimed at the potential role of *A. bisporus* in modeling meat quality.

Declaration of Interest

All authors declare that they have no conflict of interest.

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SAŽETAK

Cilj ovog istraživanja bio je provjeriti prisutnost oksidativnih enzima u četveroglavom bedrenom mišiću (m. quadriceps femoris) ovaca pasmine Lička pramenka te procijeniti učinak u hranu dodanog pripravka plemenite pečurke *Agaricus bisporus* (*A. bisporus*) na histokemijske i morfološke karakteristike mišića. Četrnaest jednogodišnjih ovaca bilo je nasumično odabrano i podijeljeno u dvije skupine (kontrolna i pokusna skupina) s po sedam životinja. Pokus je bio proveden tijekom 6 tjedana hranjenja. Kontrolna skupina bila je hranjena standardnom hranom, dok je pokusnoj skupini u standardnu hranu umješano 1,5% suhog pripravka *A. bisporus*. Tijekom cijelog pokusnog razdoblja hrana i voda bile su životinjama dostupne *ad libitum*. Uzorci m. quadriceps femoris ovaca u pokusu bili su analizirani s obzirom na veličinu i tip mišićnog vlakna, te na prisutnost oksidativnih enzima. Mišićna vlakna većih promjera i snažnije histokemijske reakcije zabilježena su u ovaca hranjenih s dodatkom *A. bisporus*. Temeljem povećane aktivnosti oksidativnih enzima, što je rezultiralo pojačanim staničnim metabolizmom i povećanom mišićnom masom ovaca hranjenih uz dodatak pripravka plemenite pečurke, možemo zaključiti da *A. bisporus* ima učinak promotora rasta.

Ključne riječi: *Agaricus bisporus*, oksidativni enzimi, ovce, Lička pramenka