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Changes in *Pleurotus ostreatus* nutritional value and heavy metal profile as a result of supplementation with nano-additive

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Abstract

The present study evaluated the effect of Lithovit-Amino25 on the nutrient profile and heavy metal composition of *Pleurotus ostreatus*. The product was tested in two doses applied at three different timings: T2: 3 g kg^{-1}/spawning, T3: 3 g kg^{-1}/after first harvest, T4: 3 g kg^{-1}/spawning and after first harvest, T5: 5 g kg^{-1}/spawning, T6: 5 g kg^{-1}/after first harvest, and T7: 5 g kg^{-1}/spawning and after first harvest. Compared to control (T1: non-treated substrate), mushrooms' fibers and carbohydrates increased in all treatments, recording the highest values in T4 (4.16%) and T3 (18.42%), respectively. Protein content was higher in mushrooms of substrates treated at spawning, with a 0.33% improvement in T5. Fat content decreased in T3, T4, T6, and T5. Total sugars decreased in mushrooms of treated substrates, and glucose was the dominant sugar in mushrooms. Fructose increased in mushrooms of T3 and T4. Calcium, iron, and potassium decreased in mushrooms of treated substrates. Sodium decreased in T3, T5, and T7, magnesium increased in T2, and phosphorus increased only in T2 and T7. Copper content of all treated mushrooms was in the standard safe limit (< 40 ppm), and it decreased in T2, T4, and T5 by around 2.5, 6.6, and 5.1 ppm, compared to control. However, zinc content increased in mushrooms of all treated substrates, and nickel and lead, increased by respective ranges of 2.8–11.88 ppm and 9.1–21 ppm, higher than the safe limits. The product presented a risk of heavy metal bioaccumulation even with a low dose.

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INTRODUCTION

Nowadays, a large amount of agro-industrial wastes are annually abandoned in the environment without any pre-treatment[1,2]. These wastes could be incorporated in mushroom production as a method to reduce their negative impacts on the environment which result from their hazardous disposal[3–5]. For instance, enormous amounts of spent mushroom substrate (SMS) resulting from mushroom cropping are discharged hazardously and need to be managed[6]. In parallel, researchers have long been utilizing SMS in mushroom production[7,8] taking advantage of its richness in lignocellulosic materials, availability, and low cost[9]. It is a nutritious substrate containing considerable amounts of minerals[10], protein and carbohydrates[11], and contains good amounts of cellulose (40%) and hemicellulose (20%)[12].

Pleurotus ostreatus ranks as the second most cultivated mushroom in the world[13]. It is valued because it is rich in protein, fibers, vitamins and (C, D and B-complex), and amino acids, and is low in calories[14,15]. The mushroom can utilize available lignocellulosic materials[16,17], such as the SMS. *Pleurotus* spp. can biodegrade SMS by producing the enzymes cellulases, hemicellulases, and ligninases[18]. The subsequent utilization of the growing substrate will result in an SMS poor in nutrients and proteins[19]. As a result, such substrates are commonly being amended with protein-rich additives to ameliorate its nutritional profile, thus ensuring higher production and quality of mushrooms[20]. Furthermore, amino acids can increase the performance of the mushroom[21]. An improvement in the biological yield of oyster mushroom was found after supplementing the SMS with 3 g kg^{-1} of a nanometric size nitrogen additive (nano-amino) applied twice during the production cycle[22]. Further, the supplement type, dose, and application timing had a major impact on the nutritional composition and heavy metal profile of *P. ostreatus* mushroom when the SMS substrate was supplemented with nano-urea[8].

Eventually, the substrate nutrient composition and properties are factors determining the mushroom nutritional composition and heavy metal profile[1,8,23]. Studying the mushrooms heavy metal profile as affected by recycled substrates and its subsequent impact on human health is taking researchers interest nowadays[24]. Consequently, the present study will showcase the effect of applying nano-amino with different doses and at separate timings during the cropping cycle on the nutrient composition and heavy metals profile of oyster mushroom cultivated on a substrate containing SMS.

MATERIALS AND METHODS

Substrate preparation and properties

The substrate used was a 1:1, w/w mixture of wheat straw and spent mushroom substrate. The latter was procured by 'Gourmet' farm (at Byblos, Lebanon) and was previously used to grow oyster mushroom. It was subjected to a sun-drying process for 1 week and then shopped for size reduction. Thereafter, the mixture of SMS and wheat straw was pasteurized for 8 h using hot water (60–65 °C) and then allowed to cool to 25 °C

for spawning. The substrate properties determined by a series of analytical tests were as follows: C:N ratio = 43:1 (determined by CHN Carlo-Erba elemental analyzer, Model 1106, Italy), moisture content: 85.6% (by Moisture Analyzer), organic matter: 82.8% dry weight (by loss of ignition over 24 h at 430 °C), pH (1:5, w/v): 5.2 (by pH meter: UltraBasic-UB10 Denver Instrument, USA), total proteins: 7.5% dry weight (by Micro-Kjeldahl method using N × 6.25[25], and total carbohydrates: 30.5% dry weight (using the Anthrone method)[26].

Substrate supplementation

Supplementation of the growing substrate applied a nitrogen-rich fertilizer (Lithovit-Amino25), containing 16 water-soluble vegetable l-amino acids, composed of calcium carbonate (50.0%), calcium oxide (28.0%), silicon dioxide (9.0%), total nitrogen (3.0%), magnesium oxide (1.8%), iron (0.5%), and manganese (0.02%)[27]. It was used in two separate doses and at three different timings. The experimental design was carried out for full factorial testing of the effect of two factors: product dose and timing of product application, through the following treatments: T2: 3 g kg^{-1}/spawning, T3: 3 g kg^{-1} /after first harvest, T4: 3 g kg^{-1} /spawning and after first harvest, T5: 5 g kg^{-1}/spawning, T6: 5 g kg^{-1}/ after first harvest, and T7: 5 g kg^{-1}/spawning and after first harvest. Each treatment was applied to 10 bags (10 replicates/treatment). To apply the nitrogenous additive at different timings, two solutions of two different concentrations (3 g L^{-1} and 5 g L^{-1}) were prepared, and from each solution 0.5 l was sprayed on the substrate according to the corresponding treatment, keeping the substrate moisture content at 60%.

Spawning and incubation

Spawning of the substrate was carried out at the 5% rate using a grain spawn of the strain M2175, procured from Mycelia Company (Deinze, Belgium)[22]. Polyethylene bags filled by the spawned substrates were then incubated at 25 °C in dark conditions. Inside the incubation room, relative humidity was maintained around 80%–90% by an ultrasonic mist maker (Hotsale 7 L h^{-1}) throughout the incubation period (14 d). At the end of the vegetative growth phase, fruit induction was triggered by reducing CO_2 levels (to 900–2,300 ppm by ventilation), lighting (using 200 lx light source), and cooling the growing room to a temperature of 15 °C. At this stage, the relative humidity was 88–90%. Regulation of room temperature and relative humidity during incubation and fruit induction applied a humidity-temperature meter (Lutron HT-3007SD).

Analytical tests

Several analytical tests served for determining the mushrooms' chemical composition, using 100 g of fresh mushrooms (pileus and stipe) of each treatment. The macro-Kjeldahl method was used to determine the total protein content with the conversion factor N × 4.38[28]. Total carbohydrates were determined using the Anthrone method[26]. Fiber analysis was carried out by applying the Weende technique[29]. After extracting a known weight of powdered mushroom sample with ethyl ether, the analysis of fat content was performed using Aldrich® Soxhlet extraction apparatus Z556203. Mushroom samples were boiled in water for 30 min to analyze soluble sugars, and 20 µl of the filtrate was then used for normal phase extraction using High Performance Liquid Chromatography at 30 °C (column NH_2 column: 250 mm × 4.5 mm ID, flow rate of 1.2 ml min^{-1}). Sugar identification applied a refractive index detector (RID), mobile phase: mixture of polar-non-polar solution, calibration: using a 2 point concentration), comparing with standards prepared from stock solution of sugars to get concentrations approximate to the sample. Mineral composition (Ca, Mg, K, Mg, Na, Fe, and Mn) was determined by adding 2.8 ml of HNO_3 (65%) to 5–6 g of samples, digesting at 150 °C for 1 h, filtrating with 100 ml of distilled water, and subjecting the filtrate to Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Phosphorus content was determined by spectrophotometry[30]. Nickel, copper, lead and zinc content were measured by Atomic Absorption Spectrophotometer (Perkin Elmer, Model Analyst 400, USA) after digesting the mushroom samples with a mixture of HNO_3, H_2SO_4, and H_2O_2 (4:1:1) (12 ml per 1 g of sample). The mixture was then boiled at 150 °C for 4 h, and diluted to 25 ml with deionized water. Similarly, a blank digest was prepared. For calibration, standard solutions were prepared by diluting stock solutions (1,000 mg L^{-1}; Sigma and Aldrich, Burlington, USA) of each metal.

Fresh samples of mushrooms were used for the analysis and results (mean values of 3 replicates ± standard deviation) were converted and expressed as percentage dry weight.

Statistical analysis

Data analysis applied the One-way ANOVA and means were compared by Duncan's multiple range test at $p < 0.05$ using SPSS25 program.

RESULTS

Results in Table 1 show that the product application resulted in a significant reduction in fat content for mushrooms of T3, T4, T6, and T7, but a significant increase in this component in mushrooms of T2 (by around 0.05% compared with control). Fiber and carbohydrates content increased significantly ($p < 0.05$) in the majority of treatments compared with control. The product applied in a dose 3 g kg^{-1} caused a higher increase in fiber content of mushrooms compared with the dose 5 g kg^{-1}; this effect was especially pronounced with the lowest product dose applied twice (T4), causing the highest fiber content in mushrooms (4.16%). Improvement in carbohydrates content was the highest with 3 g kg^{-1} nano-amino applied after first harvest (T3: 18.42%), followed by that obtained with 5 g kg^{-1} applied at spawning (T5: 10.56%). Protein content recorded a significant improvement (0.33%) in mushrooms of T5 compared to control. Total sugars including glucose and sucrose were lower in mushrooms of substrates subjected to nano-amino application. However, there was a punctual increase of fructose in mushrooms obtained in substrates treated with 3 g kg^{-1} after first harvest (T3), and at both tested timings (T4).

Findings in Table 2 showed that although it is a good source of calcium and iron, nano-amino couldn't increase these nutrients in produced mushrooms. On the contrary, calcium, iron, and also potassium was significantly ($p < 0.05$) lower in mushrooms obtained from treated substrates than in control. Further, mushrooms obtained in treated substrate had a manganese content comparable (T3, T4, T5, T6, and T7) or significantly lower (T2) than that of mushrooms obtained in control substrate. Sodium content decreased by 0.0015%, 0.0012% and 0.0010% were noted respectively in the treatments T3, T5, and T7. Overall, zinc content increased in mushrooms of all treated substrates. Magnesium content

Table 1. Composition (%fw) of *P. ostreatus* obtained from supplemented substrates.

	Fats	Fiber	Total carbohydrates	Total proteins	Total sugars	Fructose	Glucose	Sucrose
T1	0.16 ± 0.02d	2.69 ± 0.25b	4.36 ± 0.35a	2.92 ± 0.13d	0.18 ± 0.02e	0.005 ± 0.00a	0.17 ± 0.02d	0.01 ± 0.00b
T2	0.21 ± 0.01e	3.56 ± 0.02d	4.33 ± 0.07a	2.95 ± 0.04d	0.021 ± 0.00ab	0.005 ± 0.00a	0.011 ± 0.00ab	0.005 ± 0.00a
T3	0.05 ± 0.01a	3.94 ± 0.02e	18.42 ± 0.03e	2.82 ± 0.03c	0.047 ± 0.00d	0.012 ± 0.00c	0.03 ± 0.01c	0.005 ± 0.00a
T4	0.09 ± 0.02c	4.16 ± 0.01f	7.49 ± 0.02c	2.24 ± 0.02a	0.023 ± 0.00bc	0.009 ± 0.00b	0.009 ± 0.00ab	0.005 ± 0.00a
T5	0.17 ± 0.02d	3.30 ± 0.16c	10.56 ± 0.02d	3.25 ± 0.01e	0.021 ± 0.00ab	0.005 ± 0.00a	0.011 ± 0.00ab	0.005 ± 0.00a
T6	0.052 ± 0.01ab	2.43 ± 0.02a	7.10 ± 0.16b	2.23 ± 0.01a	0.025 ± 0.00c	0.005 ± 0.00a	0.015 ± 0.00b	0.005 ± 0.00a
T7	0.07 ± 0.02b	2.81 ± 0.02b	7.44 ± 0.01c	2.64 ± 0.02b	0.015 ± 0.00a	0.005 ± 0.00a	0.005 ± 0.00a	0.005 ± 0.00a
p-value								
Dose	0.00	0.00	0.00	0.09	0.44	0.00	0.02	1.00
Timing	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
Dose × Timing	0.01	0.00	0.00	0.00	0.00	0.00	0.04	1.00

T1: control, T2: 3 g kg^{-1}/spawning, T3: 3 g kg^{-1}/after first harvest, T4: 3 g kg^{-1}/spawning and after first harvest, T5: 5 g kg^{-1}/spawning, T6: 5 g kg^{-1}/after first harvest, T7: 5 g kg^{-1}/spawning and after first harvest. Means in the same column followed by different letters are significantly different at $p < 0.05$.

Table 2. Mineral composition of *P. ostreatus* mushrooms cultivated on supplemented substrates.

	Ca (%fw)	K (%fw)	Mn (ppm)	Fe (ppm)	Na (%fw)	Mg (%fw)	P (%fw)	Zn (ppm)
T1	0.0036 ± 0.00d	0.36 ± 0.04d	1.4 ± 0.00b	22.0 ± 0.00f	0.0080 ± 0.00c	0.020 ± 0.00e	0.68 ± 0.01c	42.50 ± 0.1a
T2	0.0018 ± 0.00c	0.37 ± 0.00d	0.9 ± 0.00a	14.0 ± 0.00a	0.0083 ± 0.00c	0.023 ± 0.00f	0.90 ± 0.16d	65.28 ± 0.1b
T3	0.0012 ± >0.00b	0.29 ± 0.00c	1.3 ± 0.00b	20.0 ± 0.00d	0.0065 ± 0.00a	0.015 ± 0.00b	0.64 ± 0.01bc	71.22 ± 0.1c
T4	0.0010 ± 0.00a	0.24 ± 0.00a	1.3 ± 0.00b	18.0 ± 0.00c	0.010 ± 0.00d	0.014 ± 0.00a	0.62 ± 0.01bc	72.18 ± 0.1cd
T5	0.0018 ± 0.00c	0.31 ± 0.00c	1.1 ± 0.00ab	16.0 ± 0.00b	0.0068 ± 0.00ab	0.016 ± 0.00c	0.46 ± 0.02a	46.30 ± 0.2a
T6	0.0013 ± 0.00b	0.26 ± 0.00b	1.1 ± 0.00ab	21.0 ± 0.00f	0.0079 ± 0.00c	0.015 ± 0.00b	0.57 ± 0.02b	83.10 ± 0.2e
T7	0.0014 ± 0.00b	0.30 ± 0.00c	1.1 ± 0.00ab	20.0 ± 0.00e	0.0070 ± 0.00b	0.017 ± 0.00d	0.83 ± 0.02d	76.48 ± 0.1d
p-value								
Dose	0.01	0.04	0.41	0.00	0.00	0.00	0.00	0.46
Timing	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00
Dose × Timing	0.01	0.00	0.15	0.00	0.00	0.00	0.00	0.00

T1: control, T2: 3 g kg^{-1}/spawning, T3: 3 g kg^{-1}/after first harvest, T4: 3 g kg^{-1}/spawning and after first harvest, T5: 5 g kg^{-1}/spawning, T6: 5 g kg^{-1}/after first harvest, T7: 5 g kg^{-1}/spawning and after first harvest. Means in the same column followed by different letters are significantly different at $p < 0.05$.

increased punctually in T2 by around 0.003% compared to control. Phosphorus content increased in T2 and T7, by 0.22% and 0.15% respectively.

The heavy metal analysis of mushrooms (Table 3) revealed that the copper content of all treated mushrooms met the WHO standard safe limit (2009) (< 40 ppm). Compared to control, copper content of T2, T4 and T5 mushrooms was reduced by around 2.5, 6.6, and 5.1 ppm respectively. Despite the dose and timing of supplementation, mushrooms of treated substrates had increasing and dramatic values of nickel and lead, which increased by respective ranges of 2.8–11.88 ppm (2.8–11.88 mg kg^{-1}) and 9.1–21 ppm (9.1–21 mg kg^{-1}).

Table 3. Heavy metals in *P. ostreatus* mushrooms cultivated on supplemented substrates.

	Copper (ppm)	Nickel (ppm)	Lead (ppm)
T1	13.90±0.1de	8.80±0.2a	6.20±0.1a
T2	11.38 ± 0.1c	14.70 ± 0.2e	15.30 ± 0.2b
T3	13.28 ± 0.1d	16.30 ± 0.2f	18.70 ± 0.2d
T4	7.28 ± 0.1a	12.86 ± 0.1c	19.78 ± 0.1e
T5	8.76 ± 0.1b	20.68 ± 0.1g	18.00 ± 0.2cd
T6	14.00 ± 0.2de	11.60 ± 0.2b	27.20 ± 0.1f
T7	14.22 ± 0.1e	14.28 ± 0.1d	17.66 ± 0.1c
p-value			
Dose	0.00	0.00	0.00
Timing	0.00	0.00	0.00
Dose × Timing	0.00	0.00	0.00

T1: control, T2: 3 g kg^{-1}/spawning, T3: 3 g kg^{-1}/after first harvest, T4: 3 g kg^{-1}/spawning and after first harvest, T5: 5 g kg^{-1}/spawning, T6: 5 g kg^{-1}/after first harvest, T7: 5 g kg^{-1}/spawning and after first harvest. Means in the same column followed by different letters are significantly different at $p < 0.05$.

DISCUSSION

Mushroom protein content was higher when substrates were supplemented at spawning rather than other timings. It could be that high nitrogen doses accumulating in the growing substrate could counteract the assimilation of amino acids and sugars from the substrates, causing a lower synthesis of proteins, sugars, and carbohydrates in mushrooms. The initial nitrogen level is a crucial factor for the microbiota development within the substrate[31]. When nitrogen is excessive in the substrate it plays a negative effect on the growth and development of the mycelium in the growing substrate. An increase of *P. ostreatus* protein content by 33.6% was found when the mushroom was cultivated on sugar cane bagasse supplemented with urea[32].

The total carbohydrate content of the substrate usually decreases after the first harvest. This is because fungi consume them along with other nutrients during growth[33]. Therefore, the higher carbohydrate content contained in the mushrooms of T3 and T6 (supplementation after first harvest) in comparison with control cases could be explained by the fact that the product, with high nitrogen content, has boosted the degradation of the substrate lignocellulose hence facilitating carbohydrate metabolism. Improvement in mushroom carbohydrates could be linked to a better degradation of lignin in the growing substrate. The highest decrease in substrate lignin was

reported after a low dose of nano-amino was applied at double timings during *P. ostreatus* production cycle[22].

Further, to obtain the amino-acids from substrates, the mushroom needs first to degrade the substrate protein via extracellular enzymatic secretion. The mushroom could then synthesize proteins. The product applied in the present study is initially rich in amino-acids, providing a more easily available form of amino acids compared to those obtained after the biodegradation of substrate' proteins. As a result, mushrooms treated with nano-amino at spawning had higher protein content than those treated at later stages of the production cycle. But, the double application of the product did not essentially ameliorate the protein synthesis in mushrooms, probably because of high nitrogen accumulating in the substrate and negatively affecting the mushroom growth and metabolism. Moreover, the application of nano-supplement (nano-urea) to spent mushroom substrate was reported to improve the protein content in produced mushrooms[8].

Carbohydrate foods are important source of fiber, with positive physiological effects on human health[34]. In the human body, proteins and other nitrogenous compounds are constantly broken down and contribute to the amino acid/nitrogen pool, from which precursors and amino acids are reused to produce enzymes, hormones, immune- functioning proteins, and other essential compounds[35].

Generally, mushrooms are known to have low total soluble sugar content[36]. As observed, glucose was the most abundant type of sugar found in produced mushrooms, but it was significantly reduced in all mushrooms of treated substrates compared to control. The substrate used to grow *P. ostreatus* is formed by wheat straw, containing around 36% cellulose, which, when broken down by the mushrooms' enzymes, secretes simple sugars, like glucose[37]. A lower degradation of cellulose in substrates treated with nano-amino may have caused a lower assimilation of sugars from the substrates, causing lower sugar content in mushrooms. Nano-amino application caused a lower cellulose biodegradation[22]. Further, the sugar composition of *P. ostreatus* mushrooms obtained in the present study is close to that obtained after supplementing SMS with nano-urea[8].

In general, the mineral composition of mushrooms is normally affected by the substrate's mineral profile[38]. Also, the substrates' pH may affect the heavy metal bioaccumulation and favor the absorption of certain minerals at the expense of others[39]. The product applied, initially rich in calcium carbonate ($CaCO_3$: 50%) could increase the substrate pH, resulting in a variable mineral profile of mushrooms obtained in the different treatments. The effects of nitrogen supplementation on mineral uptake levels is directly related to the substrate composition[32].

Further, it is well known that high lignin decomposition by *P. ostreatus* could be linked to a high MnP liberation in the substrate[40]. This liberation may have enhanced the MnP enzymatic activity in treated substrates richer in Mn due to nano-amino application. Manganese content in treated substrates may have been completely used by MnP at the stage of mycelial run which inhibited its translocations to mushrooms in a further stage. The substrate supplemented at spawning with a product dose of 3 g kg^{-1} showed higher lignin degradation compared to non-treated substrate[22]. A reduction of food sodium content is favored for blood pressure control[1] (https://meadowmushrooms.co.nz/storage/wysiwyg/files/final-nutritional-analysis-of-meadow-mushrooms-a-summary.pdf).

The competition between metals in soil affects the absorption of some of these metals by wild mushrooms[41]. This may suggest a serious metal competition occurring in treated substrates and favoring the absorption of zinc at the expense of calcium and iron from the substrates. *P. ostreatus* is rich in phosphorus and phosphorus-rich foods are good contributors in human nutrition[14]. However, high levels may inhibit calcium absorption causing weak bones, itchy skin, and joints pain that can lead to mineral bone disorders in chronic kidney disease[42]. Foods rich in protein and carbohydrates were associated with zinc accumulation[43]. In the current study, all treated mushrooms with nano-amino showed high zinc levels above the safe level (60 ppm) set by the WHO[44] except when the growing substrate was supplemented at spawning with a dose of 5 g kg^{-1} (T5).

Heavy metal concentrations in edible and non-edible mushrooms are associated with mineral substrates or heavily contaminated areas such as large cities and industrial sites[45]. Oyster mushroom absorbs heavy metals from the substrate through its spacious mycelium[46]. Certain metals, such as Ca, Cu, Fe, K, Mg, Mn, Na, Ni, and Zn are biologically active in fungi[47]. High nickel levels in mushrooms could lead to serious toxicity[48]. Effectively, the levels of nickel detected in mushrooms from treated substrates were higher than the safe range of 0.05–5 ppm given for plant foods (https://nap.nationalacademies.org/read/20096/chapter/2). Moreover, lead content in control mushrooms was higher than that previously reported by Quarcoo & Adotey[46] (0.04 mg kg^{-1}) and the values recommended by the EU commission (https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2001R0466:20060701:EN:PDF) and WHO[44] (0.3 mg kg^{-1} and 2 mg kg^{-1}, respectively). Ca, Fe, K, Mn, and P are required for normal human physiological function, but prolonged overexposure to Cu and Pb may cause neurological dysfunction or overt disease. High levels of Zn, Ni, and Cu are for instance neurotoxic and may lead to seizures[49]. The mushrooms' heavy metals profile obtained in treated substrates is of concern. The exceptional reduction in Cu content in a few cases of treated mushrooms might have occurred because of the competition posed by Ni, Pb, and Zn, as suggested previously by Sassine et al.[8].

CONCLUSIONS

Investigating the nano-amino effect on *P. ostreatus* nutritional attributes showed that such a treatment could be beneficial causing a general improvement of proteins, carbohydrates, and fiber content, reduction in total sugars, coupled with punctual phosphorus increase and sodium decrease in mushrooms. Using the product once in low or high dose seems to be more advantageous than twice for carbohydrates and protein metabolism. However, even with the lowest dose applied, a risk of nickel and lead accumulation was observed suggesting that the product may have been better tested in lower doses.

Conflict of interest

The authors declare that they have no conflict of interest.

Dates

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