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The impact of polystyrene consumption by edible insects *Tenebrio molitor* and *Zophobas morio* on their nutritional value, cytotoxicity, and oxidative stress parameters

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ABSTRACT

The objective of this study was to determine of nutritional value, *in vitro* cytotoxicity, and oxidative stress parameters in cells of selected insect species (*Tenebrio molitor* and *Zophobas morio*) after 30 days of Styrofoam consumption. Furthermore, part of our research is also a consumer survey on the willingness to eat insects fed with Styrofoam (EPS 80). Mealworms fed with Styrofoam were determined to have higher protein (48.66 ± 0.92%) and ash content (4.81 ± 0.22%) with reduced fat (24.05 ± 0.55%) and carbohydrate content (2.95 ± 0.15%) than insects with a conventional diet (48.66 ± 0.92, 2.82 ± 0.12, 43.74 ± 0.77, and 4.78 ± 0.18, respectively) while in the case of superworms, no significant difference in nutrient composition was observed. Moreover, Styrofoam has no influence on the health status of gut cells in examined insects. Additionally, in studied concentrations of insects extracts standardized for protein replacement of the traditional insect diet with polystyrene foam did not increase the cytotoxic properties.

1. Introduction

In recent years, insect consumption, termed entomophagy, has been a very popular topic among researchers for at least several important reasons. Insect consumption is promoted primarily due to its high nutritional value. An increasing number of researchers emphasize that insect farming may allow a means to address the growing protein demand by humans worldwide (Liu, Li, & Gómez, 2019; Nowak, Persijn, Rittenschober, & Charrondiere, 2016; Zielińska, Baraniak, Karaś, Rybczyńska, & Jakubczyk, 2015). Moreover, insects farming produces less greenhouse gases than livestock, additionally require much less land, feed, and water (Oonincx & de Boer, 2012). Furthermore, insects are considered as a source of bioactive components such as bioactive peptides or antioxidant enzymes (Mlcek, Borkovcova, & Bednarova, 2014). In many European countries insect breeding and processing is still in its infancy. Nevertheless, there are signs that insect-based food consumption is coming to Europe and is likely to become more acceptable in the future (Raheem, Raposo, et al., 2019). A number of strategies are proposed to overcome the challenges of accepting insects as food (Raheem, Carrascosa, et al., 2019). All the positive aspects of insect farming are in favour of their consumption by a human. However, the use of insects is also considered in other aspects and areas of life.

Besides examining insects as food, researchers from China (Y. Yang, Yang, et al., 2015a) have proven that the larvae of mealworm (*Tenebrio molitor*) have the ability to utilize expanded polystyrene commonly known as Styrofoam. This is possible due to bacteria living in their digestive tract *Exiguobacterium* sp. strain YT2 (Y. Yang, Yang, et al., 2015b). This study confirmed the conversion of ingested polystyrene

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into CO_2 and biomass. This finding is even more important considering that mealworms are pests and are very easy to grow. They can be easily bred on wheat bran, or wastes from vegetables and fruits such as potato, cabbage, carrots, or apple (Y. Yang, Yang, et al., 2015a). Moreover, they reported that the larvae of waxworms (*Plodia interpunctella*) were capable of chewing and eating polystyrene films due to the presence of two bacterial strains in their gut capable of degrading polystyrene i.e., *Enterobacter asburiae* YT1 and *Bacillus* sp. YP1 (J. Yang, Yang, Wu, Zhao, & Jiang, 2014; Y. Yang, Chen, Wu, Zhao, & Yang, 2015). The authors admit that the inspiration for this research was an observation that some mandibulate insects and stored-product insect pests can chew and eat plastic packages (Riudavets, Salas, & Pons, 2007).

This topic has been continued by many other researchers confirming these surprising reports (Kiliç, 2018; Kosewska, Kosewska, Przemieniecki, & Sienkiewicz, 2019; Nukmal, Umar, Puspita Amanda, & Kanedi, 2018; Przemieniecki, Kosewska, Ciesielski, & Kosewska, 2019; Stewart, 2019; Yang, Brandon, et al., 2018; Yang, Wu, et al., 2018; Bożek, Hanus-Lorenz, & Rybak, 2017; Leluk, Hanus-Lorenz, Rybak, & Bożek, 2017). Furthermore, Yang, Brandon, et al. (2018) proved that polystyrene degradation rates are significantly enhanced by supplementing the diet with a conventional source of nutrition and then insects can reproduce and produce a second generation that can degrade polystyrene. In addition, further species that digest Styrofoam have been confirmed, such as superworm (*Zophobas atratus*) (Yang, Wang, & Xia, 2019) and *Zophobas morio* (Miao & Zhang, 2010), dark mealworms (*Tenebrio obscurus*) (Peng et al., 2019), red flour beetle (*Tribolium castaneum*) (Fabreag & Familara, 2019), caterpillars of the wax moth (*Galleria mellonella*) (Bombelli, Howe, & Bertocchini, 2017), lesser waxworm (*Achroia grisella*) (Kundungal, Gangarapu, Sarangapani, Patchaiyappan, & Devipriya, 2019). Moreover, other research confirms that insects degrade more other types of plastic wastes such as polyethylene (Bombelli et al., 2017; Brandon et al., 2018; Chalup et al., 2018), rubber (Aboelkheir, Visconte, Oliveira, Toledo Filho, & Souza, 2019), or polyvinyl chloride (PVC) and polylactide (PLA) (Bożek et al., 2017). These studies confirmed that the plastic-eating insects are commonplace in nature and they move beyond one particular species.

This finding is very important due to the problem of environmental pollution from plastic waste. Polystyrene wastes result from the commercial use of Styrofoam in popular food packages such as coffee cups and food trays. Polystyrene is a major pollutant of water reservoirs such as rivers, lakes, and oceans, and thus water organisms (Jang et al., 2016; Stewart, 2019). Despite efforts to reduce the production of plastic bags and containers, this problem of global pollution is still increasing. It is widely documented that plastic pollutions have harmful impacts in many areas such as marine wildlife or biological diversity as well as human health (Chen, Kuo, Lee, & Liu, 2018). There is no agreed data on the time it takes for plastics to degrade, but it can be hundreds or thousands of years (Verma, Vinoda, Papireddy, & Gowda, 2016). Therefore, the problem of plastic waste management becomes very important.

Utilization of plastic by insects may be a breakthrough in the aspect of the Styrofoam utilization, but it raises further questions. It is interesting how the consumption of polystyrene by insects affects their health or nutrient properties. Whether such larvae could subsequently be consumed by animals or humans. If the utilization of polystyrene foam does not have any negative impact on insect organisms, they could be further used as food and therefore their breeding would have a double benefit. We hypothesize that it is possible and insects utilizing polystyrene foam can be used as human food without any negative consequences for health.

In answering these questions the aim of our study was the determination of nutritional value, *in vitro* cytotoxicity, and oxidative stress parameters of insects after 30 days of Styrofoam consumption. Furthermore, part of our research is also a consumer survey on the willingness to eat insects fed with Styrofoam. We selected two insect species for this research: the larvae of *Tenebrio molitor* with a widely proven ability to utilize polystyrene (S. S. Yang, Wu, et al., 2018; Y. Yang et al., 2019; Y. Yang, Yang, et al., 2015b) and the larvae of *Zophobas morio* from the same family of beetles Tenebrionidae which is also being tested previously (Miao & Zhang, 2010; Yin, Zhou, Wang, & Huang, 2018).

2. Materials and methods

2.1. Materials

The mealworms *Tenebrio molitor* (Linnaeus, Coleoptera: Tenebrionidae) (larvae), and superworms *Zophobas morio* (Fabricius, Coleoptera: Tenebrionidae) were purchased from Bugstore (Krakow, Poland).

2.2. Styrofoam-feeding test

The insects underwent a two-week adaptation, during which they were kept at a temperature of 23 °C and 45% humidity and fed with wheat bran, and then the mealworms and superworms were divided into two groups – one of them was fed on Styrofoam (Styrofoam EPS 80 $\lambda = 0.038$, Swisspor, Poland) and the second on wheat bran purchased from the local store (Kupiec, Poland). Access to feed was unlimited. After 30 days of breeding, all individuals of these species were fasted for approximately 48 h to clear their gastrointestinal tract of any residual food.

2.3. Determination of the nutrient composition

The protein was determined using the Kjeldahl method (Tecator Kjeltec Auto 1030 Analyzer, Foss, Denmark) (AOAC, 2010), and the N conversion factor was 4.76 as indicated Janssen et al. (Janssen, Vincken, van den Broek, Fogliano, & Lakemond, 2017). The fat percentage was calculated by drying fats by extraction in a Soxhlet (Tecator Soxtec System HT 1043, Foss, Denmark) using hexane (AOAC, 2010). The moisture content was determined using the moisture analyzer (Radwag WPS 50SX, Poland), and the ash content was determined by drying the sample in a muffle oven (REFA, Poland) at 500 °C for 5 h (AOAC, 2010). Carbohydrates were determined by difference, by the following formula: 100 - (weight in grams [protein + fat + moisture + ash] in 100 g of edible insects). The conversion method was used for the determination of the nutritional value (European Parliment, 2011). Results were shown as mean ± SEM from 3 independent experiments (n = 9).

2.4. In vitro cytotoxicity assays

The studies assessed the impact of insect homogenates on the number of endothelial cells of the HECa10 line. Briefly, 500 mg of lyophilized insects were homogenized with a mortar in liquid nitrogen and next dissolved in 5 mL of phosphate buffered saline (PBS) solution. (stock 100 mg of insect extract/mL). After filtration (syringe filters, 0.22 μm) total protein concentration of extracts was evaluated (Bradfords method, Merck, Poznań Poland). Insect extracts (IE) then were standardized (stock 1 mg of insect protein/mL), apportioned (250 μL/probe) and stored in −80 °C until analysis. Thawing-freezing cycles were avoided. In a day of analysis, stock of IE was thawed and a series of dilutions were made.

2.4.1. Cell culture

The tests were performed on – mouse endothelial HECa10 cell line which was isolated from peripheral lymph nodes. HECa10 cells were immortalized by cationic liposome transfection. Cells have the characteristics of endothelial cells: they produce an enzyme that converts angiotensin and factor VIII antigen (Bizourane, Mitterrand, Monsigny, & Kieda, 1993). HECa10 cells were grown in DMEM with 10% FBS (FBS – fetal bovine serum) with the addition of antibiotics (Penicillin 100U,

Streptomycin 100 μg/mL), all bought from Life Technologies, Warsaw, Poland. The cells were maintained under standard culture conditions (37 °C, 95% humidity, 5% CO_2) under aseptic conditions. The cells in continuous growth were passaged after obtaining 80–90% confluency using 0.25% trypsin. Cells for the research came from 7 to 15 passage.

2.4.2. Proliferation assays

The cells (in log phase growth stage) were harvested (0.25% trypsin, 5 min, 37 °C, Life Technologies, Warsaw, Poland), centrifuged (300 × g, 5 min.), resuspended in culture medium (DMEM, 4.5 g/mL glucose with L-glutamine, 10% FBS with antibiotic) to the final density of 1×10^5 cells/mL and seeded into a 96-well plate (1×10^4 cells/well). Twenty-four hours after seeding, cells were washed twice with PBS and 10 μL of appropriate insert extracts were added to the culture medium for obtaining a final concentration of 1, 5, 10, 50, or 100 μg IE/mL of culture medium.

2.4.2.1. MTT assay. MTT assay was performed as previously described (Rokicki et al., 2014). Briefly, twenty-four hours after IE addition the culture medium was discarded and a fresh culture medium with MTT (0.5 mg/mL) was added. After 4-hour incubation (37 °C, 5% CO_2, <90% humidity) the medium was discarded, cells were flushed twice with PBS, and 100 μL of dimethyl sulfoxide (DMSO) was added to all the wells to dissolve the formazan crystals. The absorbance was measured at 570 nm by FLUOstar Omega reader (BMG Labtech GmbH, Ortenberg, Germany). Results were shown as mean ± SEM from 3 independent experiments (n = 12).

2.4.2.2. Neutral red. NR assay as performed as previously described (Leśniak et al., 2018). Briefly, after IE addition the culture medium was discarded and fresh culture medium with Neutral Red (25 mg/ml; Sigma Aldrich, Poland) was added directly to the wells. After 2 h of incubation (37 °C, 5% CO_2, <90% humidity) the medium was discarded and cells were rinsed 3 times with PBS. To dissolve the NR, cells were rinsed with 100 μL of rinsing solution (1% acetic acid, 50% ethanol, 49% water solution all from Sigma Aldrich, Poznań, Poland). The absorbance was measured at 570 nm with FLUOstar Omega reader. Results were shown as mean ± SEM from 3 independent experiments (n = 12).

2.4.2.3. Sulforhodamine B assay. SRB assay as performed as previously described (Leśniak et al., 2018). Briefly, twenty-four hours after IE addition the culture medium was discarded, cells were rinsed twice with PBS and cells were fixed with 50% trichloroacetic acid (Sigma Aldrich, Poland; 100 μL per well, 1 h, 4 °C). Then cells were washed 3 times with water, air dried and 100 μL of sulforhodamine B (0.4% in 1% acetic acid; Sigma Aldrich, Poznań, Poland) was added. After 30 min incubation cells were washed 4 times with 1% acetic acid, air dried and 10 mM Trisma®base (100 μL/well; Sigma Aldrich, Poland) was added to dissolve the SRB dye. The fluorescence was measured at excitation 570 nm (±10) and emission 590 nm (±10) using FLUOstar Omega reader. Results were shown as mean ± SEM from 3 independent experiments (n = 12).

2.4.3. Lactate dehydrogenase (LDH) assay

LDH is a cytosolic enzyme present in many cell types. This enzyme catalyzes the transformation lactate to pyruvate by reducing NAD^+ to NADH. After membrane damage, cellular enzyme is released into the culture medium. NADH is reduced used to reduce the tetrazolium salt (INT) to a red formazan product that can be measured at 490 nm. The amount of formazan is directly proportional to the amount LDH released into the medium, and indirectly indicates the number of damaged cells.

LDH assay was performed in accordance with manufacturer procedure (Pierce™ LDH Cytotoxicity Assay Kit, Thermo Scientific™, Warsaw, Poland). Twenty-four hours after IE addition the culture medium was collected to the tubes, in which determination of LDH activity was performed. The absorbance of red formazan was measured using FLUOstar Omega reader at 490 nm and 680 nm (background signal) and calculated as 490 nm minus 680 nm absorbance. Results were shown as mean ± SEM from 3 independent experiments (n = 12).

2.5. Parameters of oxidative stress in the gastrointestinal tract

2.5.1. Insects preparation

10 specimens from each test group were anesthetized on ice, prepared from each gastrointestinal tract in their entirety and placed in eppendorf tube with 400 μL of 0.1 M PBS buffer (pH 7.4) cooled on ice. The prepared tissue was gently homogenized in a ball homogenizer (Minilys®, Bertin Technologies) for 30 s to obtain cell suspension. The obtained homogenate was filtered and used for further analysis.

2.5.2. Detection of dead cells

To detect cells in various stages of apoptosis the Muse® Annexin V & Dead Cell Kit was used (Millipore, Billerica, MA, USA). Briefly, 50 μL of the homogenate was mixed with 50 μL of the annexin V reagent. The reaction mixture was incubated at 20 °C for 20 min. A flow cytometer Muse® Cell Analyzer (Millipore, Billerica, MA, USA) was used to make the determinations. Results were shown as mean ± SEM from 10 independent experiments (n = 10).

2.5.3. The viability and total cell count

To determine viability and total cell count the Muse® Count and Viability Kit were used (Millipore, Billerica, MA, USA). Briefly, 25 μL of the homogenate was mixed with 225 μL of the count and viability reagent. The reaction mixture was incubated at 20 °C for 5 min. A flow cytometer Muse® Cell Analyzer (Millipore, Billerica, MA, USA) was used to make the determinations. Results were shown as mean ± SEM from 10 independent experiments (n = 10).

2.5.4. Measurement of reactive oxygen species (ROS) in cells

The Muse® Oxidative Stress Kit (Millipore, Billerica, MA, USA) was used for the measurements of reactive oxygen species (ROS), namely superoxide radicals in cells undergoing oxidative stress. Briefly, 10 μL of the homogenate was mixed with 190 μL of the reagent working solution. The reaction mixture was incubated at 37 °C for 30 min. A flow cytometer Muse® Cell Analyzer (Millipore, Billerica, MA, USA) was used to make the determinations. Results were shown as mean ± SEM from 10 independent experiments (n = 10).

2.6. Consumer study

The experiment was based on a survey conducted on 285 subjects. Participants were randomly recruited from an online access panel. The survey consisted of two parts: specifications and basic questions. The participants were asked about the sex, age, and willingness to eat insects if they were available on the market. We identified two age groups: 18–29 years old, and over 30 years of age. The oldest respondent was 55 years old. Among the respondents participating in the survey, 78.9% are women (n = 225) and 21.1% men (n = 60). The majority were aged 18–29 (87.02%, n = 248). 63.5% of respondents (n = 181) had no experience in eating insects, while 36.14% consciously ate insects in the past (n = 103). The survey consisted of a set of items scored on a 5-point Likert scale ranging from “strongly disagree” to “strongly agree”:

1) I know the source and method of feeding animals whose meat I eat.
2) I would be interested in what insects are being fed that I would like to eat.
3) Insects fed Styrofoam seem to be uneatable.
4) The addition of Styrofoam to insect feed makes me a greater aversion to their consumption.
5) I would eat insects fed Styrofoam if the results of scientific research indicated that it is safe for human health.

Participation in the study was voluntary and was not associated with obtaining compensation.

2.7. Statistical analysis

Statistical analysis was performed using unpaired t-tests and one-or two-way analysis of the variance, followed by the Tukey test or *t*-Student correction (in the case of a normal distribution) or non-parametric U Mann–Whitney, Kołmogorow–Smirnow, Levene, and Brown – Forsyth tests (in the case of abnormal distribution). Assessment of the distribution of data was evaluated using the Shapiro–Wilk test. GraphPad Prism software (ver. 6; GraphPad Software, Inc., La Jolla, CA, USA) and Statistica (ver. 13,1, StatSoft Inc. Krakow, Poland) were used. $P < 0.05$ was a statistically significant difference. A one-way ANOVA test was used in the questionnaire analysis (gender and eating insect experience differences). To calculate the effect of interaction of age, gender and experience with insect's consumption we used two-way ANOVA with interactions.

3. Results and discussion

3.1. Nutrient composition

The nutritional composition and energy content of insects is listed in Table 1. Comparing the nutritional composition of mealworm (*T. molitor*) fed with Styrofoam and the control sample we can observe statistically significant differences. Mealworms fed with Styrofoam were determined to have higher protein (48.66 ± 0.92%) and ash content (4.81 ± 0.22%) with reduced fat (24.05 ± 0.55%) and carbohydrate content (2.95 ± 0.15%) than insects with a conventional diet (48.66 ± 0.92, 2.82 ± 0.12, 43.74 ± 0.77, and 4.78 ± 0.18, respectively). Therefore, mealworms fed with Styrofoam were also characterized by lower energy content. The conclusion is that the consumption of Styrofoam by insects improves their nutritional value by reducing fat and carbohydrates content while at the same time increasing protein content. Bożek et al. (2017) were conducted biochemical assays in mealworms and they confirmed that stored energy by mealworms in the form of lipids and carbohydrates is used for keeping the basic metabolic rates. They observed a reduction of fat and carbohydrates content in polystyrene-eating insects. Furthermore, their studies show that protein levels were not significantly dependent on the food type.

Yang et al. (Y. Yang, Yang, et al., 2015a) have proven that within a 16 day test period, 47.7% of the ingested Styrofoam carbon was converted into CO_2 and the residue (ca. 49.2%) was egested as fecula with a limited fraction incorporated into biomass. Moreover, they confirmed that the polystyren after mineralization to CO_2 was incorporated into lipids; hence, digestion of PS contribute to the energy needs of the mealworms. In turn, Bożek et al. (Bożek et al., 2017) was observed the mass loss of analyzed insects which indicates that polystyrene is not a sufficient source of energy for them. They suggest also that higher amount of protein in larvae fed polystyrene may be a result of sparing the energy as a form of adaptation to the difficult conditions and food unavailability.

In turn, in the case of superworms, no significant difference in nutrient composition was observed. Therefore, studied insect species have different reactions to lack of access to their normal feed for one month, and for superworms the consumption of polystyrene is less affecting the organism. Based on these conclusions, it can be presumed that superworms could feed on polystyrene longer than mealworms and could therefore utilize larger quantities of Styrofoam. Furthermore, the method of disposal of polystyrene by *Z. morio* is unclear – it is not confirmed whether this is the same proces as for *T. molitor*.

Table 1
Comparison of nutritional composition and energy content in the dry matter of edible insects.

	Studied species			
	*Tenebrio molitor*control	*Tenebrio molitor* fed on Styrofoam	*Zophobas morio*control	*Zophobas morio* fed on Styrofoam
Protein (g/100 g d.w.)	37.06 ± 0.92^{b}	51.93 ± 0.98^{a}	48.72 ± 0.89^{A}	47.71 ± 0.78^{A}
Fat (g/100 g d.w.)	43.74 ± 0.77^{a}	24.05 ± 0.55^{b}	31.25 ± 0.63^{A}	32.88 ± 0.67^{A}
Ash (g/100 g d.w.)	2.82 ± 0.12^{b}	4.81 ± 0.22^{a}	2.63 ± 0.1^{A}	2.51 ± 0.15^{A}
Carbohydrates (g/100 g d.w.)	16.38 ± 0.18^{b}	19.21 ± 0.15^{a}	17.4 ± 0.12^{A}	16.9 ± 0.17^{A}
Dry mass (g/100 g f.w.)	36.29 ± 0.62^{a}	36.66 ± 0.9^{a}	48.93 ± 0.53^{A}	48.29 ± 0.66^{A}
Energy (kJ/100 g)	2527 ± 12.8^{a}	2099 ± 11.2^{b}	2280 ± 9.8^{A}	2315 ± 10.5^{A}
Energy (kcal/100 g)	607 ± 4.3^{a}	501 ± 3.9^{b}	546 ± 4.2^{A}	554 ± 4.4^{A}

Different letters among the same species indicate significant difference ($\alpha = 0.05$) between control and fed on Styrofoam ($n = 9$).

3.2. In vitro cytotoxicity

There are only several works, which described effect of *T. molitor* extracts on cell cytotoxicity. Lee et al. in *in vitro* study on different cell lines that larval extract from mealworm (0.08 – 2 mg/ml) showed no effect of cell number measured by MTT in normal hepatocyte cell line and increased number of cells in normal cardiomyocyte cell line (Lee et al., 2015). What was interesting cell numbers of human cancer cell lines isolated from liver (Hep3B, and SK-HEP-1 cell lines) was significantly decreased after treatment of water larval extract from *T. molitor* in all tested range (0.5 – 2 mg/ml). Similar results were obtained by Wu et al., (2020). Normal murine fibroblast L929 cell line (cell line recommended for testing cytotoxicity of medical devices) was not affected after *T. molitor* oil extract in added in the range of 1–10 mg/ml. In contrast cancer cell lines: Caco-2, HepG2, HeLa was significantly affected by extract from mealworm larvae. Han et al., (2014) showed no mutagenic or clastogenic character of *T. molitor* larvae in rat feed orally per 28-day extracts of the worm. Also all tested parameters of the rat (body weight, parameters of blood system, urine system and biochemical evaluation in serum) was unaffected by the worm feed. Moreover, *T. molitor* extracts may improve some parameters of life. Pessina et al. demonstrated significant reduction in blood pleasure, heart rate and coronary perfusion pressure, as well as an increase in red blood cell glutathione/glutathione disulphide ratio in spontaneously hypertensive rats (Pessina et al., 2020). These findings suggested, mealworm extracts should be non-cytotoxic for normal cell lines and organism. We also observed this relationship in *in vitro study*. Generally, in present work all tested concentrations for *T. molitor* (1–100 μg/mL of protein in IE), no significant differences in the number of cells measured by NR, SRB tests were noticed in IE fed control diet. In MTT assay significant reduction of cells number was seen in all tested range and in significant increase of LDH activity in concentration 10–100 μg/mL of protein in IE of insect fed control diet (Fig. 1). Extract from insects supplemented with foamed polystyrene did not show significant cytotoxic properties, and what seems more, the extracts obtained in this way had lower cytotoxic properties than control one (Fig. 1). Some slight static differences were noted between the groups in the MTT test (without a clear trend) and NR (with a clear trend to increase cell numbers).

For *Z. morio*, as in the case of mealworm (*T. molitor*), data from all tests indicate that in the tested concentrations (1–100 μg/mL of protein in the extract) there are no significant differences in the number of cells measured by MTT, NR, SRB, and LDH tests. Extract from Styrofoam supplemented insect did not show significant cytotoxic properties (except the higher concentration of IE in MTT assay, Fig. 2), and was characterized by lower cytotoxic properties (visible especially in the

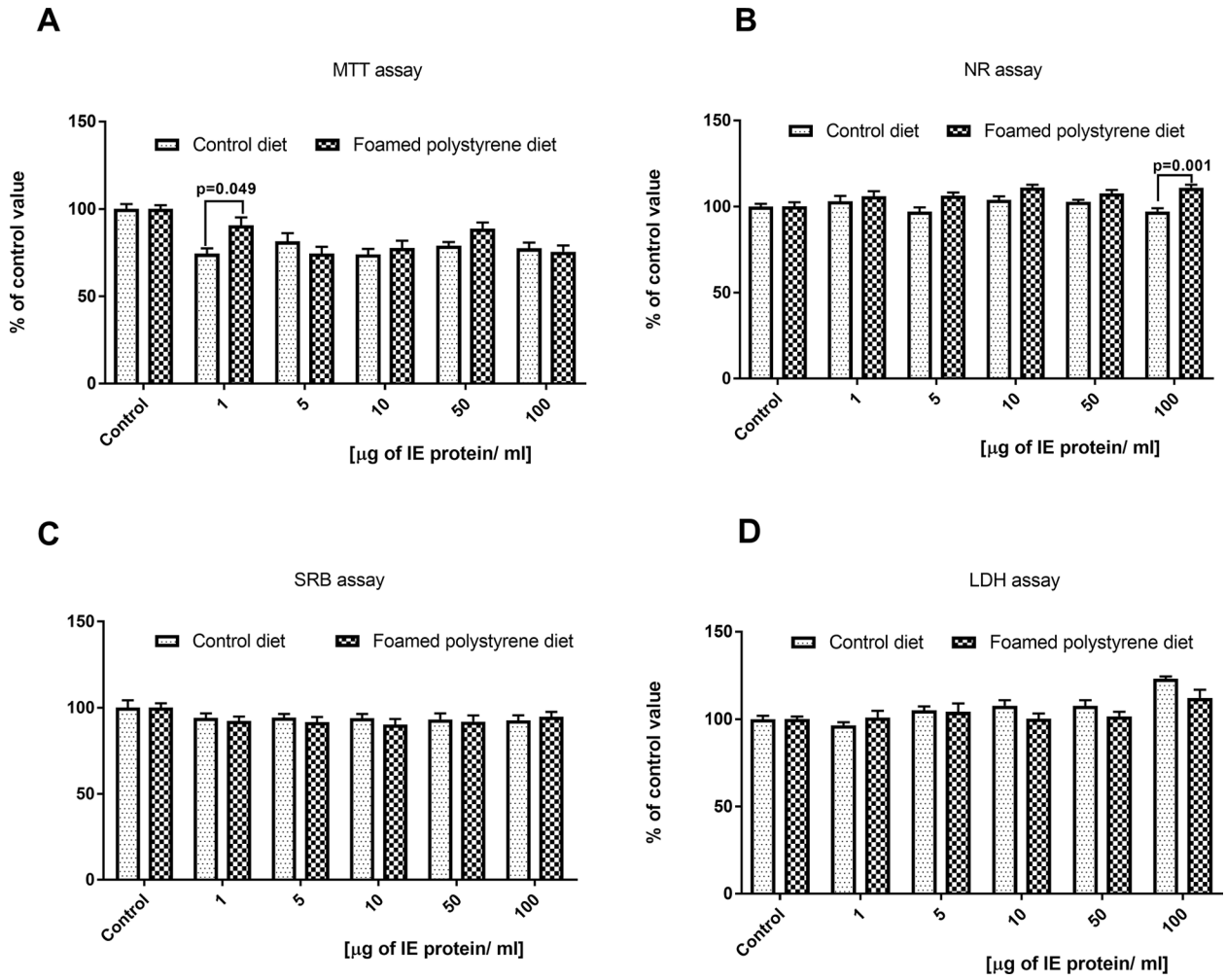

Fig. 1. Effect of extracts from mealworm fed control or polystyrene diet on cells number measured by MTT, NR, SRB and LDH assay. Analysis performed after 24 h after addition of mealworm extract in concentration: 1, 5, 10, 50 and 100 µg / ml of protein in IE**. A** - MTT assay; B - NR assay; C - SRB assay; D - LDH assay. The results are from 3 independent experiments (n = 12).

LDH test, statistically significant changes in concentration: 50–100 µg protein of IE/mL). There were also some differences between the groups in the MTT test (probably not associated with cell number but with the effect of IE from Styrofoam group on redox potential) as well as NR and LDH: significant increase of cell count in NR assay (100 µg protein of IE/mL) associated with the reduction of LDH releases (50–100 µg protein of IE/mL, Fig. 2).

Results from *Z. morio* presented here are in agreement with other studies. Yusof et al. showed that in low doses of *Z. morio* etanol extract (below 100 µg/mL) there was no cytotoxicity of Vero cells (normal kidney epithelial cells extracted from an African green monkey) (Yusof, Chowdhury, Faruck, & Sulaiman, 2017). Interestingly, authors observed cytotoxicity of the extract on human breast cancer MCF-7 cell line. The same group found that ethanolic extracts of *Z. morio* had higher IC50 than isopropanolic on MCF-7 cell line, 1.7 mg/ml and 0.7 mg/ml respectively (Chowdhury, Yusof, Faruck, & Sulaiman, 2015).

In summary, studied concentrations of insect extracts standardized for protein (1–100 µg/mL protein in the extract), replacement of the traditional insect diet with polystyrene foam did not increase the cytotoxic properties. What was interesting extracts from insects fed foamed polystyrene exhibited lower cytotoxicity compared to insects fed a control diet. It had to been noted, that we used four different tests for measure potential of cytotoxicity and overall this complex analysis should be analyzed as a whole. The obtained results seem very promising. We are aware, however, that we performed the analysis only one type of cells (endothelial) therefore studies should be repeated on other types of cells (e.g. fibroblasts, epithelial cells) so that *in vitro* toxic effects of insect homogenates can be excluded.

3.3. Health status of cells: Oxidative stress in the gastrointestinal tract

Styrofoam does not influence the health status of gut cells in examined insects *T. molitor* and *Z. morio* (Fig. 3, Fig. 4). Both species present no statistical differences among exanimated groups.

Although there are no statistical differences in terms of measured parameters, in *Z. morio* cell viability, a trend can be observed: a greater share of dead cells in the Styrofoam group comparing to control. Similar results in *T. molitor* groups can be seen, but the differences are slighter (Fig. 3). The participation of dead cells in both species reveals, that in general *T. molitor* presents higher cell viability, than *Z. morio* and *Z. morio* has greater sensitivity to Styrofoam than *T. molitor*.

There are no differences in apoptotic cells share between the control and Styrofoam groups, but it is worth to mention, that *T. molitor* has a higher level of apoptosis than *Z. morio*.

Both species present a very similar level of reactive oxygen species. The ROS (+) share is a very low, comparable to the level of the control.

The flow cytometry results indicate that feeding insects with Styrofoam has no impact on free radical generation in the digestive tract, which is the primary organ directly exposed to the potential risk factor.

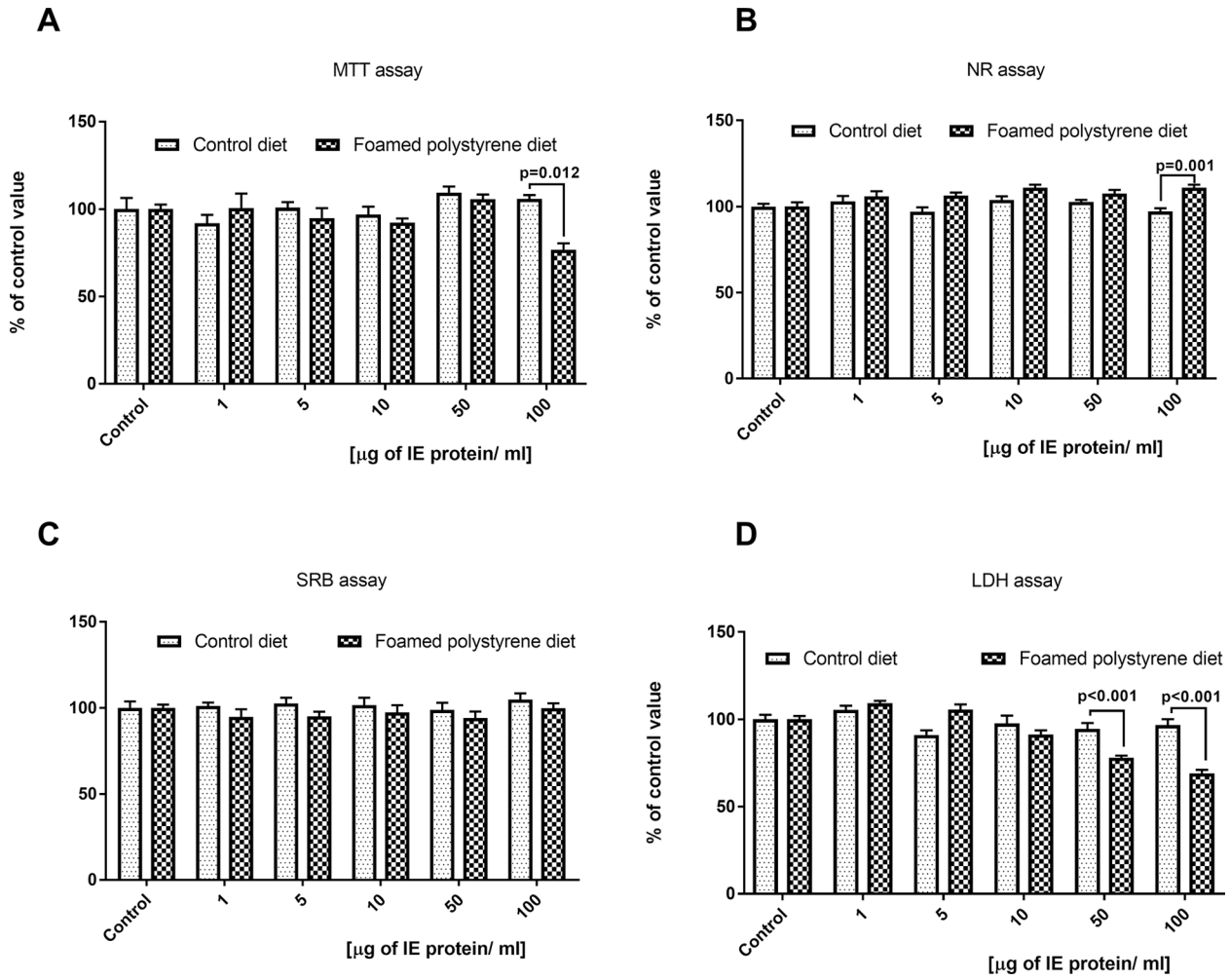

Fig. 2. Effect of extracts from *Zophobas morio* fed control or polystyrene diet on cells number measured by MTT, NR, SRB and LDH assay. Analysis performed after 24 h after addition of mealworm extract in concentration: 1, 5, 10, 50 and 100 μg / ml of protein in IE. A - MTT assay; B - NR assay; C - SRB assay; D - LDH assay. The results are from 3 independent experiments (n = 12).

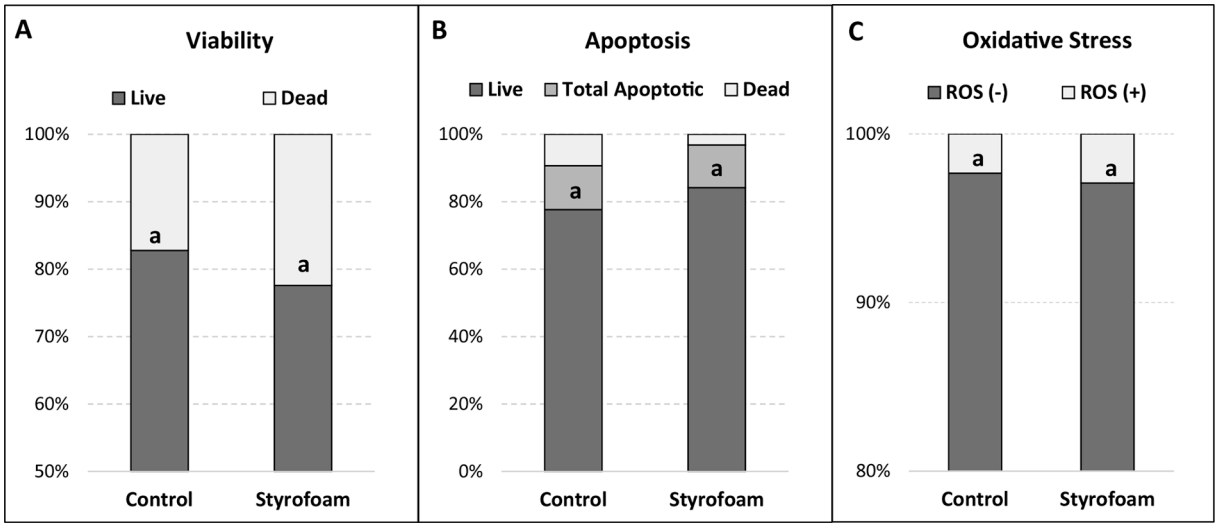

Fig. 3. Health status of gut cells in *Tenebrio molitor* fed with styrofoam. A - viability of cells, B - level of apoptosis C - Reactive oxygen species. Significant differences measured by ANOVA (post-hoc LSD test $p < 0.05$). Different letters denote differences among experimental groups.

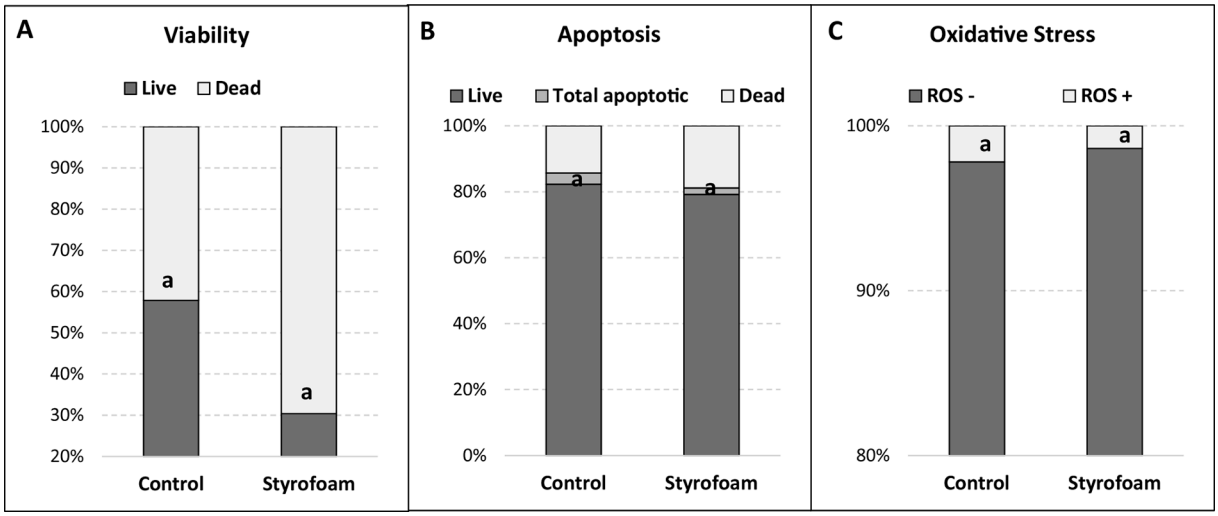

Fig. 4. Health status of gut cells in *Zophobas morio* fed with styrofoam. A - viability of cells, B - level of apoptosis C - Reactive oxygen species. Significant differences measured by ANOVA (post-hoc LSD test $p < 0.05$). Different letters denote differences among experimental groups.

3.4. Consumer study

The mean values of answers to the statements made in the survey were in the range of 2.97 to 3.75 ($p = 0.00$) (Table 2). This indicates that the respondents do not reject the possibility of eating insects fed on polystyrene foam, and even their attitude is closer to positive opinions on this issue.

Females have significantly more negative attitudes about the idea of eating insects fed with Styrofoam (3.79) and the addition of Styrofoam to the diet of insects makes them more aversive to entomophagy than males (3.88 for females, 3.26 for males) (Table 2). In general, there was no difference between men and women in their knowledge of the origin of the animals they eat as well as in their interest in what insects were fed to them for later consumption. It is worth noting, that it is men who would be much more willing to eat insects fed on polystyrene if scientific research confirms their safety than women (mean 3.58, 2.92, respectively, $p < 0.01$).

There were no differences between the compared age grups in their answers while the experience of respondents in eating insects had a significant effect on every statement made in the survey. People who have not eaten insects before claimed that insects fed with Styrofoam are uneatable and it makes them aversive about entomophagy than before. On the other hand, respondents who already eat insects were more positive about the statement of eating insects fed with polystyrene in comparison to people with no entomophagy experience (mean 3.90, 2.58, respectively, $p < 0.05$). We have found no interactions between age, gender and past experience with entomophagy ($F = 0.17$, $df = 5$, $p = 0.973$; $F = 0.65$, $df = 5$, $p = 0.659$, respeqtively).

The results of these observations confirm that the human consumption of insects that degrade polystyrene is not excluded provided that their consumption is safe.

4. Conclusions

Styrofoam utilization is an important worldwide problem therefore, the ability of insects to digest it seems very important. However, the impact of polystyrene consumption by insects on their organisms is equally significant. Nevertheless, the results of our determinations are very promising. Generally, Styrofoam consumption and degradation does not influence the health status of studied insect species *T. molitor* and *Z. morio*. These results encourage further analysis to confirm the absence of toxicity of polystyrene to insect organisms that degrade it. An unequivocal statement on the safety of insects that degrade Styrofoam would allow its subsequent use in animal or human feeding. However,

Table 2
Results of statements concerning Styrofoam feeding of insects (1 = strongly disagree; 5 = strongly agree) on the total sample and by gender and the willingness to eat insects.

Statement	Mean (sd)	DF	*t*-Value	Male mean (sd)	Female mean (sd)	F-Value	p	Willingness to eat insects mean (sd)	Unwillingness to eat insectsmean (sd)	F-Value	p
I know the source and method of feeding animals whose meat I eat	2.97 (0.07)	277	41.13	2.87 (0.17)	3.00 (0.08)	0.58	0.449	3.20(0.12)	2.83(0.09)	5.91	0.016***
I would be interested in what insects are being fed that I would like to eat	3.65 (0.08)	284	44.31	3.62 (0.19)	3.66 (0.09)	0.04	0.839	4.13(0.10)	3.39(0.11)	19.69	0.000*
Insects fed Styrofoam seem to be uneatable	3.68 (0.07)	284	53.24	3.28 (0.16)	3.79 (0.07)	9.21	0.003**	3.34(0.12)	3.88(0.08)	14.58	0.000*
The addition of Styrofoam to insect feed makes me a greater aversion to their consumption	3.75 (0.07)	284	50.46	3.26 (0.17)	3.88 (0.08)	11.74	0.001*	3.47(0.13)	3.91(0.09)	8.28	0.004***
I would eat insects fed Styrofoam if the results of scientific research indicated that it is safe for human health	3.06 (0.09)	284	34.09	3.58 (0.20)	2.92 (0.10)	9.35	0.002**	3.90(0.13)	2.58(0.11)	60.31	0.000*

DF – Degree of Freedom; Signif. codes: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$, Tukey test.

the studies that have been carried out confirm the assumption that insects degrading Styrofoam may be safe for human consumption. It seems quite controversial currently, but it is a great opportunity to concurrently use the potential of edible insects in many areas of life.

CRediT authorship contribution statement

Ewelina Zielińska: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Visualization, Writing - original draft. **Damian Zieliński:** Conceptualization, Formal analysis, Investigation, Methodology, Resources, Visualization, Writing - original draft. **Anna Jakubczyk:** Conceptualization, Methodology, Supervision, Validation. **Monika Karaś:** Conceptualization, Methodology, Supervision, Validation. **Urszula Pankiewicz:** Methodology, Resources, Validation. **Barbara Flasz:** Data curation, Formal analysis, Investigation, Methodology, Resources, Visualization, Writing - original draft. **Marta Dziewięcka:** Methodology, Investigation, Visualization, Writing - original draft. **Sławomir Lewicki:** Data curation, Formal analysis, Investigation, Methodology, Resources, Visualization, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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