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IN OVO STIMULATION WITH *LACTOBACILLUS* SYNBIOTIC INDUCES CHANGES IN HEPATIC PROTEINS INVOLVED IN THE *DE NOVO* BIOSYNTHESIS OF PURINES, ENERGETIC METABOLISM AND STRESS PREVENTION IN BROILER CHICKENS

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Abstract

In ovo technology ensures the delivery of a bioactive substance that supports the organism at the stage of embryonic development via modulation of intestinal microbiota. Many years of research on injection during egg incubation have shown that the administration of the synbiotic on day 12 affects the improvement of production and physiological parameters, improves the microbiological profile of the intestines, and induces molecular changes. In our previous analyses at the protein level, we have demonstrated a global picture of changes in the liver proteome in response to in ovo stimulation with synbiotic by using MALDI-TOF MS. The aim of this study was to extend the analysis of proteomic changes after in ovo administration of the synbiotic using ultra-high performance liquid chromatography combined with electrospray ionization quadrupole time-of-flight mass spectrometry to reveal much more liver proteins which may change in response to stimulation. The injection was performed on the day 12 of egg incubation by administering the synbiotic Lactobacillus plantarum with raffinose family oligosaccharides. The proteins were isolated from livers collected post-mortem from 21-day-old Cobb 500FF broiler chickens. Significant down-regulation of a fold change was demonstrated for ACAC, GART, FDPS and AIRC. A numerically low but statistically significant up-regulation has been demonstrated for HSP90B1, ENO1 and PRDX. This study expands our understanding of the molecular effects of in ovo delivered synbiotic in the liver of chickens and provides another premise that alterations in gut microbiota play a key role in maintaining proper liver functions via modulating the gut–liver axis.
Key words: in ovo, liver, proteome, synbiotic

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Introduction

Multifaceted studies on the effects of *in ovo* injection of *Lactobacillus*-based synbiotic on the day 12 of egg incubation have led to a number of significant discoveries. As it has been proven, the administration of *Lactobacillus plantarum* with raffinose family oligosaccharides (RFO) in one dose causes independent action of both components in the digestive system of the host. The prebiotic component stimulates the development of the host's intestinal microbiota, thereby improving the intestinal microbiological balance. On the other hand, probiotic microorganisms colonize the digestive system. Such a relationship has been defined as the synergism of a synbiotic in relation to the host, and not the components in relation to each other. Considering the extensive mechanism of action of the substances on the host, significant communication on the gut–liver axis at the molecular level was to be expected. Based on the conducted research in this area, we have shown that the administration of the above-mentioned synbiotic influences changes in expression and methylation of metabolic and immunological genes (Siwek et al., 2018; Dunislawska et al., 2020). In addition, our previous analyses at the protein level indicated that the synbiotic may have profound effects on the functioning of the mitochondria in the liver, has the potential to accelerate major energy-supplying metabolic pathways, and leads to a reduction in liver lipid accumulation (Dunislawska et al., 2021). Our analysis clearly indicated that it is necessary to extend the proteomic analysis with more sophisticated proteomic techniques. Hence, the aim of this study was to extend the analysis of proteomic changes after *in ovo* administration of the synbiotic using ultra-high performance liquid chromatography combined with electrospray ionization quadrupole time-of-flight mass spectrometry (UHPLC-ESI-QTOF MS) to reveal much more liver proteins which may change in response to stimulation.

Material and methods

Experimental setup

The detailed experimental setup was described by Dunislawska et al. (2021). 5,850 eggs from Cobb 500 FF broiler chickens (Cobb Vantress B.V., Boxmeer, Holland) were incubated in a commercial hatchery (Drobex-Agro, Solec Kujawski, Poland). Eggs were incubated at 37.8°C and relative humidity between 61 and 63%. On day 12 of incubation, eggs were randomly distributed to control and experimental groups. Eggs in control group (C) were injected into air chamber with 0.2. mL physiological saline (0.9%), experimental group (S) was injected with 0.2 mL of synbiotic (*Lactobacillus plantarum* combined with RFO). The synbiotic consisted of RFO (2 mg/egg) isolated from seeds of lupin *Lupinus luteus* L. cv. Lord and *Lactobacillus plantarum* IBB3036 (10^5 bacteria cfu/egg). After hatching, the roosters were intended for rearing. The main experiment was conducted in a commercial farm (Piast, Olszowa, Experimental Unit 0161, Poland). Birds had *ad libitum* access to fresh drinking water and were fed from day 1 of age. On the day 21 of rearing, slaughter was performed, after which the liver (n=8/group) was collected for further analysis. Method of synbiotic selection, dose optimization and production data were described in Dunislawska et al. (2017). Animal use for experiments was approved by the Local Ethical Committee for Animal Experimentation, University of Science and Technology, Bydgoszcz, Poland (Approval No. 36/2012 on 12 July 2012).

Protein isolation

The detailed method of protein isolation is presented in Dunislawska et al. (2021). Liver samples (n=8/group) were homogenized in lysis buffer containing urea, thiourea, CHAPS, DTT, Biolyte, protease inhibitor cocktail, and nuclease at using a mechanical homogenizer.

Insoluble tissue debris was removed by centrifugation and the supernatant containing the solubilized liver proteins was collected. Isolated proteins were intended for analysis by a nano UHPLC-ESI-QTOF MS.

Nano UHPLC-ESI-QTOF MS system, conditions and data processing

All samples were analyzed as described previously (Adamiak et al., 2022). Briefly, normalized amount of 20 µg of proteins from each whole liver tissue lysate was precipitated using ice-cold (–20°C) Acetonitrile (LC-MS hyper grade ACN, Merck) in volume 1:4 ratio. After precipitation samples were centrifuged and the protein pellet was dissolved in 40mM ammonium bicarbonate. Reduction and alkylation were carried out using 500 mM DTT and 1 M IAA. Proteins in solution were digested for 16 h in the presence of Trypsin Gold (Promega) at 37°C and 600 RPM. Digested samples were diluted with 0.1% formic acid (ThermoFisher) and centrifuged (+2°C, 30 min., 18 000 × g) before nano UHPLC separation.

LC-MS analysis was carried out with the use of nano UHPLC (nanoElute, Bruker) coupled by CaptiveSpray to ESI-Q-TOF mass spectrometer (Compact, Bruker). Two-column separation method was used, i.e., pre-column (300 µm × 5 mm, C18 PepMap 100, 5 µm, 100Å, Thermo Scientific) and Aurora separation column (75 µm × 250 mm, C18 1.6 µm) in gradient 2% B to 35% B in 90 min with the 300 nL/min flow rate. In total 200 ng of polypeptides mixture were injected to the column. Following mobile phases were used: A – 0.1% formic acid in water; B – 0.1% formic acid in ACN.

The collected spectra in the positive polarity mode in the range 150–2200 m/z, with the acquisition frequency of the 4 Hz spectrum and the autoMS/MS system were analyzed and calibrated (Na Formate) in DataAnalysis software (Bruker) and then, after extracting the peak list, identified in ProteinScape (Bruker) using the MASCOT server. Proteins were identified using the online SwissProt and NCBIprot databases, and their annotation and biological significance were determined using UniProt, Reactome.org, String.org, and KEGG.

Data normalization and statistical analysis

Proteomic data was transformed by logarithmation. Two-stage normalization was performed by average (1) and by slope (2) in accordance with the methodology proposed by Aguilan et al. (2020). The fold change (FC) of protein abundance was calculated after normalization and imputation of the data using the formula: experimental group/control. In order to check the statistical differences between the control and experimental groups, a non-parametric Mann-Whitney unpaired test was performed using GraphPad Prism 9 for Windows (GraphPad Software, San Diego, California USA). A statistically significant difference was considered at $P<0.05$. The volcano plot showing the results was prepared using GraphPad Prism 9 for Windows (GraphPad Software, San Diego, California USA).

Results

Changes in the abundance of protein expression for the experimental group versus the control one are shown in the volcano plot in Figure 1. Significant down-regulation of a fold change was demonstrated for: ACAC (FC = –2.18), GART (FC = –1.9), FDPS (FC = –1.85) and AIRC (FC = –0.98). A numerically low but statistically significant up-regulation has been demonstrated for: HSP90B1 (FC = 0.28), ENO1 (FC = 0.34) and PRDX1 (FC = 0.42).

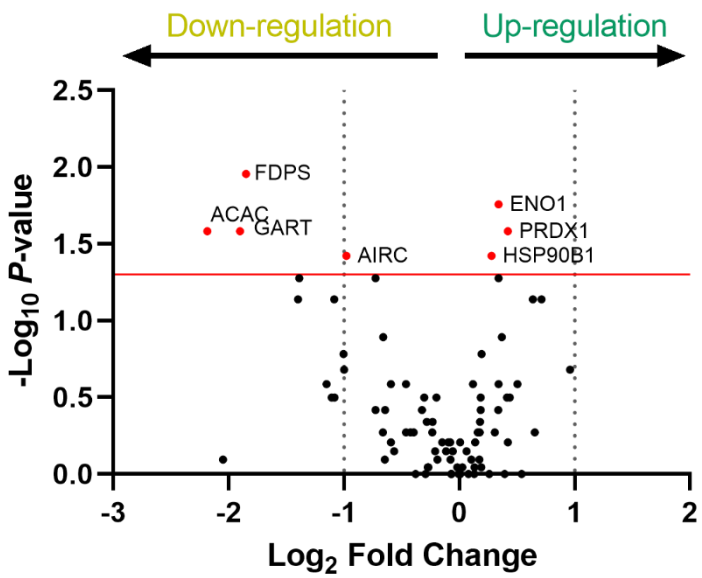

Figure 1. The volcano plot shows the results for the proteome in liver tissue of chicks stimulated with synbiotic on the day 12 of the egg incubation. The x-axis shows Log_2 fold change (FC) (experimental group/control group), the y-axis shows $-Log_{10}$ for the P value (Mann-Whitney test) for the proteome determined in chicken liver tissue after *in ovo* stimulation with synbiotic. Proteins with a statistically significant FC were separated by a solid red line for $P<0.05$, which gives a cut-off point on the y-axis at the level of 1.3; additionally, these proteins were marked as red dots. The dashed gray lines on the x-axis represent the cutoff for a one-time FC (negative and positive)

Discussion

This study extends our previous research in which the effects of *in ovo* delivered *Lactobacillus* synbiotic on the hepatic proteome changes of broiler chickens were assessed using two-dimensional gel electrophoresis (Dunislawska et al., 2021). Here, an UHPLC-ESI-QTOF MS-based approach was applied to further discriminate protein modifications in broiler liver tissue upon *in ovo* stimulation with *Lactobacillus*-based synbiotic. Two significantly down-regulated genes GART and AIRC, belong to a multi-enzyme complex known as a purinosome, which is involved in *de novo* purine biosynthesis (Zhao et al., 2013). Based on the results, we found that the two key proteins associated with the purinosome formation were down-regulated in liver cells following *in ovo* administration of the *Lactobacillus*-based synbiotic. Purines and purinergic signalling are crucial for various cellular processes, including maintenance of energy homoeostasis and nucleotide synthesis and they can be either synthesized directly in the cells or utilized from the diet (Huang et al., 2021). Previous study has demonstrated that activation of the salvage pathway, which utilizes exogenous purine, suppresses the ATP-consuming *de novo* purine synthesis pathway (Lee et al., 2020). Decreased expression of proteins associated with purinosome formation found in the current study could be attributed to the increased gut microbiota diversity. This is further supported by evidence from a recent study where increased *Lactobacillus* abundance was shown to be negatively correlated with uric acid concentration in mouse intestine, suggesting its direct role in dietary purine catabolism (Wu et al., 2020). The current and our previous study have shown a moderate increase (0.28) of HSP90B1 expression and more pronounced (0.63) down-regulation of HSP70 in the liver of birds from the experimental group (Dunislawska et al., 2021). It should be pointed out that these two molecular chaperones have been recently linked to purinosome formation as a decreased expression of HSP70 or HSP90 were shown to lead to the dramatic decreases in the purinosome content in the HeLa cells (French et al., 2013).

Moreover, *in ovo* delivered *Lactobacillus* synbiotic was shown to suppress lipogenesis in the liver of broiler chickens as evidenced by a reduced abundance of ACAC protein involved in fatty acid synthesis as well as down-expression of farnesyl pyrophosphate synthase (FDPS), one of an obligate intermediate for *de novo* cholesterol synthesis. This result ties well with previous studies wherein mRNA expression levels of key mediators associated with the novo lipogenesis were shown to be down-regulated in the liver of broiler fed with a diet supplemented with probiotics or a dietary fiber, guar gum (Zhang et al., 2019). Emerging evidence from this study suggests a key role of short chain fatty acids (SCFAs) in maintaining liver lipid regulation through modulating the gut–liver axis. Additional *in vitro* studies, performed by the same authors, showed SCFAs-induced glucagon-like peptide-1 (GLP-1) secretion in intestinal epithelial cells, which in turn has been shown to directly reduce hepatic fat synthesis by activating AMP-activated protein kinase/Acetyl CoA carboxylase pathway in primary hepatocytes (Zhang et al., 2019). In our study we have also observed an increased hepatic expression of ENO1, a cytoplasmic protein implicated in modulating cell viability and glycolysis levels as well an increased abundance of PRDX1, which is considered as a main cytoprotective protein showing antioxidative potential. Other proteomic results were broadly in line with our findings showing that dietary supplementation of probiotics (*Enterococcus faecium*) or prebiotics (4% of dried chicory root) may alter the carbohydrate metabolic pathway and enhance protection against oxidative stress in the livers of broiler chickens (Zheng et al., 2016) and growing pigs (Lepczyński et al., 2017). Overall, this study expands our understanding of the molecular effects of *in ovo* delivered *Lactobacillus* synbiotic in the livers of broiler chickens and provides another premise that alterations in gut microbiota play a key role in maintaining proper liver functions via modulating the gut–liver axis.

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STYMULACJA *IN OVO* SYNBIOTYKIEM *LACTOBACILLUS* INDUKUJE ZMIANY W BIAŁKACH WĄTROBOWYCH ZAANGAŻOWANYCH W BIOSYNTEZĘ PURYN *DE NOVO,* METABOLIZM ENERGETYCZNY I ZAPOBIEGANIE STRESOWI U KURCZĄT BROJLERÓW

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STRESZCZENIE

Technologia *in ovo* zapewnia dostarczenie bioaktywnej substancji, która wspiera organizm na etapie rozwoju embrionalnego poprzez modulację mikrobioty jelitowej. Wieloletnie badania nad iniekcją podczas inkubacji jaj wykazały, że podanie synbiotyku w 12. dniu wpływa na poprawę parametrów produkcyjnych i fizjologicznych, poprawia profil mikrobiologiczny jelit oraz indukuje zmiany molekularne. W naszych wcześniejszych analizach na poziomie białkowym wykazaliśmy globalny obraz zmian w proteomie wątroby w odpowiedzi na stymulację *in ovo* synbiotykiem przy użyciu techniki MALDI-TOF MS. Celem tego badania było rozszerzenie analizy zmian proteomicznych po podaniu synbiotyku *in ovo* przy użyciu ultra-wysokosprawnej chromatografii cieczowej w połączeniu z tandemową spektrometrią

mas z jonizacją typu electrospray i analizatorami kwadrupol i TOF, aby ujawnić znacznie więcej białek wątroby, które mogą ulec zmianie w odpowiedzi na stymulację. Iniekcję przeprowadzono w 12. dniu inkubacji jaj, podając synbiotyk *Lactobacillus plantarum* z oligosacharydami z rodziny rafinozy. Białka wyizolowano z wątroby pobranej pośmiertnie od 21-dniowych brojlerów kurzych Cobb 500FF. Wykazano znaczącą redukcję zmiany krotności dla ACAC, GART, FDPS i AIRC. Liczbowo niskie, ale statystycznie istotne zwiększenie regulacji wykazano dla HSP90B1, ENO1 i PRDX. Badanie to poszerza naszą wiedzę na temat molekularnych efektów synbiotyku dostarczanego *in ovo* w wątrobie kurcząt i dostarcza kolejnej przesłanki, że zmiany w mikrobiocie jelitowej odgrywają kluczową rolę w utrzymaniu prawidłowych funkcji wątroby poprzez modulację osi jelito–wątroba.

Słowa kluczowe: *in ovo*, wątroba, proteom, synbiotyk