

# Protein replacement with dried distiller's grain with solubles (DDGS) in practical diet of common carp (*Cyprinus carpio*)

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**Abstract.** A 12-week feeding trial was conducted to evaluate the effects of feeding corn DDGS to juvenile common carp (*Cyprinus carpio* L.) (initial weight 63.1±11.4 g) in controlled rearing conditions with stocking density of 30 ind/m<sup>3</sup>. Three iso-nitrogenous (35%) and iso-lipidic diets (6%) were formulated with DDGS inclusion levels of 0%, 20% and 40%. Growth parameters, nutrient and protein utilization, body composition, biochemical and histological parameters were examined at the end of trial. *C. carpio* demonstrated significant advantages for groups fed with DDGS-containing feeds compared to the control in most of the measured parameters (FBW, WG, FCR, PER, PPV). Protein efficiency ratios were significantly different between all groups, and the highest efficiency (1.68 g/g) was found in the DDGS 40 group. The examined fish did not show differences in biometric indices and in biochemical parameters related to metabolic injuries, irrespective of the dietary composition. The highest deposition for oleic acid (18:1n-9) in the liver was observed in the DDGS 00 group by contributing to up 40% of the lipids. Our results showed that conventional sources of plant protein can be substituted with DDGS (up to 40%) without negative consequences on the growth and health of common carp.

**Key Words:** bioethanol by-product, nutrition, biochemistry, histology, experimental feed.

**Kivonat.** 12 hetes etetési kísérletet végeztünk kukorica DDGS tartalmú összetett takarmány tesztelésé céljából ponty (*Cyprinus carpio* L.) ivadékkal (kezdő súly 63,1±11,4 g). A kísérletet zárt, kontrollált halnevelő rendszerben végeztük, ahol a telepítési sűrűség 30 db/m<sup>3</sup> volt. Az etetéshez három különböző DDGS tartalmú (0%, 20 %, 40%) tápot terveztünk azonos fehérje (35%) és zsír (6%) értékekkel. A kísérlet végén értékeltük a növekedési, takarmány és fehérjehasznosítási paramétereket, testösszetételt, biokémiai és hisztológia elváltozásokat. A DDGS 40 csoport legtöbb vizsgálati paraméterében (FBW, WG, FCR, SGR, PER, PPV) szignifikánsan különbözött a DDGS 00 kontroll csoporttól. A fehérjehasznosítás mindegyik csoport között szignifikánsan eltért, a legmagasabb érték (1,68 g/g) a DDGS 40 csoportnál volt mérhető. A biometriai paraméterek és az anyagcsere betegségekre utaló vérkémiai paraméterek nem különböztek a takarmányok hatására. Jelentős zsírideponálás volt megfigyelhető a DDGS 00 csoport májszövetében, ahol a zsírsav profil 40%-át képezte az olajsav (18:1n-9). Eredményeink alapján elmondható, hogy a *C. carpio* nevelésre használt takarmányok növényifehérje forrásai lecserélhetők akár 40%-ban DDGS-re a termelési és élettani mutatók változása nélkül.

**Kulcsszavak:** kukorica törköly, haltakarmányozás, biokémiai, hisztológia, közönséges ponty.

**Introduction.** In the past decades, studies have focused on evaluating a variety of different plant ingredients to meet the nutritional needs of fish. Protein resources such as soybean (Hardy 1982; Pongmaneerat & Watanabe 1993; Oliva-Teles et al 1994; Kaushik et al 1995; Webster et al 1992; El-Dahhar & El-Shazly 1993), maize gluten meal (Wu et al 1995), lupins (Fontainhas-Fernandes et al 1999), rapeseed (Davies et al 1990), cottonseed meal (Dadgar et al 2010; Rinchard et al 2002), corn gluten (Moyano et al 1992; Robaina et al 1997; Jahanbakhshi et al 2012) and canola meal (Hardy & Sullivan 1983; Lim et al 1998; Abbas et al 2008; Yurkowski et al 1978; Thiessen et al 2004) have already been successfully tested as fishmeal alternatives. However, the inclusion of plant ingredients brings its own set of challenges related to product quality and environmental impacts (Hardy 2010).

When formulating a diet, the availability and price of different feedstuffs should also be taken into account. Local by-products from food, fermentation and the pharmaceutical industry can be of particular interest for a sustainable and energy-efficient perspective due to lower production costs and less environmental pressure. In this sense, DDGS (Dried Distiller's Grain with Solubles), a by-product from bioethanol production, can make a significant contribution to the sustainable development of EU aquaculture. Its high amounts of energy, medium protein quantity, digestible fiber and accessible phosphorous enable the preparation of sustainable fish feeds with a high nutritional value. Moreover, DDGS has an additional advantage over other plant feed ingredients, namely its lack of antinutrient factors (Makkar 2012). Fermentation makes the nutrients in DDGS twice or three times more concentrated (Belyea et al 2004), although the composition always depends on the source of the grain (Belyea et al 2010).

The incorporation of DDGS in the feeds of swine, poultry and other monogastric animals is well studied and commonly applied (Wadhwa & Bakshi 2016). However, on a global level, there is a knowledge gap in the usefulness of DDGS for aquafeed, and it only has been tested on a few species such as rainbow trout (*Oncorhynchus mykiss*) (Cheng & Hardy 2004; Øverland et al 2013; Welker et al 2014), channel catfish (*Ictalurus punctatus*) (Lim et al 2009; Li et al 2011b), Nile tilapia (*Oreochromis niloticus*) (Schaeffer et al 2009, 2012; Li et al 2011a; Khalil & El-sharkawy 2013), hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) (Coyle et al 2004), European seabass (*Dicentrarchus labrax*), meagre (*Argyrosomus regius*) (Magalhães et al 2015), sunshine bass (*Morone chrysops* × *M. saxatilis*) (Thompson et al 2008) and turbot (*Scophthalmus maximus*) (Diógenes et al 2018). In some of these cases, the results are really promising, albeit with a remarkable variation.

*C. carpio* is one of the most important fish species in freshwater aquaculture, especially in Europe and East Asia. Because of its high importance as food and game fish, its production exceeds 4 million tons per annum (FAO 2018). In contrast to traditional pond aquaculture where fish are fed only on a supplementary basis with grains, monoculture based on reliable and manageable dry feeding is currently receiving increased interest. High-quality but cheap dry feeds can enhance the intensification of aquaculture and improve profitability through cheaper production.

To improve the production rates of these valuable species, it would be important to develop cheap dry feeds using readily available and sustainable ingredients such as DDGS. The digestibility of this ingredient has been determined in the frame of a previous work on *C. carpio* and was 94.42% and 76.23% for the apparent digestibility coefficient of protein and dry matter (Sandor et al 2016). In this context, the aim of the present study was to evaluate the effects of high DDGS inclusion levels on growth performance, nutrient utilization, biochemical indices and molecular markers in *C. carpio* in order to promote sustainable dry feeding of this species.

## Material and Method

**Experimental animals and design.** The *C. carpio* juveniles were provided by own production. The fish previously were kept in earthen ponds using traditional carp production technology. Before experiment the fish were kept in a quarantine indoor tank system for 3 weeks. During the quarantine period, they were treated according to Németh et al (2013) with DETOX SA (ORPC, Italy) and Dimilin® 25WP (Chemtura, The Netherlands) to disinfect parasites (Yasuno & Satake 1990; Bouboulis et al 2004). Initial body weight of fish was  $63.1 \pm 11.4$  g (average  $\pm$  STD,  $n = 180$ ). The fish were stocked in 1 m<sup>3</sup> tanks with a water temperature of  $24 \pm 0.5^\circ\text{C}$ ; the experiment lasted for 12 weeks and each treatment was triplicated. The experimental groups were settled to their tanks randomly, using random number generator. Additional oxygen was dissolved to the tanks by cones and sprayers. The fish were fed manually three times a day in equal quantities. The daily amount of feed was calculated based on 3.0–3.5% of tank biomass, which was corrected bi-weekly according to the attained weight when all fish were carefully anesthetized and measured. Water parameters were monitored twice a week. Dissolved

oxygen values were measured continuously and were always above 80%; pH was 8.4-8.5 and ammonium levels were  $<0.15 \text{ mg L}^{-1}$ .

**Experimental diets.** The Pannonia Ethanol corn-based DDGS, was produced via dry milling in Dunaföldvár, Hungary from locally produced corn and free from any antibiotics and GMO ingredients. Its nutritional profile was as follows: crude protein 27%, crude fat 9%, crude fiber 10%, crude ash content 4-6%, starch 3% and P content 7-10  $\text{g kg}^{-1}$ , calculated on dry matter basis. Lysine, methionine, tryptophan and threonine contents were present at low concentrations in regard to the nutritional demands of the fish (NRC 2011). Three isonitrogenous ( $\sim 35\%$ ) and isolipidic ( $\sim 6\%$ ) experimental diets suitable for carp species were planned and produced by Nagyhegyesi Takarmány Zrt., Nagyhegyes, Hungary. The capacity of the commercial extruder was 1 metric ton per hour. DDGS inclusion was set to three different levels (0% as DDGS 00, 20% as DDGS 20 and 40% as DDGS 40). All diets were based on terrestrial plants and industrial by-products, without any fish meal. Hemp seed oil was the lipid source because of its optimal n-6:n-3 (3:1) fatty acid ratio and its beneficial effects on fish health (Da Porto et al 2012). Synthetic lysine and methionine were added to the experimental feeds to balance the essential amino acid ratio. Formulation and composition of the diets are presented in Table 1. The physical characteristic of the feeds was semi-floating with 4.5 mm diameters.

**Sampling.** At the end of the feeding period, all fish were starved for 24 hours when the final body weights were measured (Lines & Spence 2012). Five fish were selected randomly and over-anesthetized by a Norcaicum-based solution (Matuk 1987). One mL of blood was taken from the caudal vein of two fish, using heparinized needles and syringes. Blood samples were put into heparinized microcentrifuge tubes and centrifuged at 1,400 g and 4,000 rpm for 20 minutes at  $4^{\circ}\text{C}$ . After centrifugation, blood plasma was collected and stored at  $-20^{\circ}\text{C}$  for further analysis. The fish were dissected to measure biometrical indices (condition factor, hepatosomatic index, viscerosomatic index) and to take samples from the liver, muscle, head kidney and mid-gut. The remained three whole fish were frozen at  $-20^{\circ}\text{C}$  until further proximate composition analysis.

**Feed and fish analysis.** The chemical compositions of fish and feed were analyzed by standard methods of the AOAC (1998) (Table 1). Crude protein was determined by the Kjeldahl-method, using a digestion block (Kjeldaltherm, Gerhardt, Germany) and the distillation procedure (Vapodest 30, Gerhardt, Germany) and calculated as  $\text{N} \times 6.25$ . The crude fat was assigned by a Soxtherm unit (Gerhardt, Germany), using petroleum ether (boiling point:  $40-60^{\circ}\text{C}$ ) as solvent. Dry matter and ash content were determined by gravimetry after drying at  $105^{\circ}\text{C}$  and burning at  $550^{\circ}\text{C}$  in a furnace. Crude fiber was measured with a Gerhardt Crude Fiber installation (Gerhardt, Germany), using sulfuric acid and potassium-hydroxide as digestive solvents. The experimental diet's total carbohydrate (TC) and gross energy (GE) values were calculated as  $\text{TC} = 100 - (\text{crude protein} + \text{crude fat} + \text{crude fiber} + \text{ash})$ , with  $\text{GE} = \text{values of carbohydrates, proteins and lipids of } 17.2, 23.6 \text{ and } 39.5 \text{ KJ g}^{-1}$ , respectively (Halver & Hardy 2002). The fatty acid compositions of fish and feed samples were analyzed by capillary gas chromatography. The lipids were extracted with chloroform/methanol (2:1, by vol) and the extracts purified according to the method by Folch et al (1957). After esterification, fatty acid methyl esters (FAME) were separated on a fused silica capillary column (DB-225) in an AGILENT (HP) gas chromatograph system (type „6890N“) equipped with flame ionization detector. Amino acids were measured via the ISO 13903:2005 standard method at the National Food Chain Safety Office's accredited laboratory.

Table 1

Formulation (%), proximate composition (% as is basis), amino acid profile and fatty acid profile of the experimental diets used

<i>Ingredients (%)</i>	<i>DDGS 00</i>	<i>DDGS 20</i>	<i>DDGS 40</i>
Soybean meal	50.4	28.4	30.1
Meat meal (poultry)	26.5	33.3	23.3
Maize	17.0	14.7	3.0
DDGS <sup>1</sup>	0.0	20.0	40.0
Hemp seed oil	3.8	1.0	0.0
Vitamin and mineral premix <sup>2</sup>	2.0	2.0	2.0
CaCO <sub>3</sub>	0.0	0.0	1.0
DL-methionine	0.1	0.1	0.1
L-lizine	0.0	0.2	0.2
Choline Chloride	0.2	0.2	0.2
<b>Proximate analysis</b>			
Dry matter (%)	90.49	89.82	90.39
Crude protein (%)	36.92	34.49	32.78
Crude fat (%)	5.66	4.20	4.10
Crude fibre (%)	3.12	3.67	4.53
Crude ash (%)	11.26	10.72	9.20
Gross Energy (MJ/kg)	17.63	17.05	17.02
<b>Amino acid profile (m/m%)</b>			
<u>Essential amino acids</u>			
Arginine	2.43	2.09	1.79
Histidine	1.42	0.90	0.82
Isoleucine	1.47	1.32	1.30
Leucine	2.62	2.54	2.59
Lysine	1.94	1.74	1.54
Methionine	0.52	0.59	0.56
Cystine	0.34	0.43	0.45
Phenylalanine	1.35	1.41	1.47
Threonine	1.99	2.08	2.02
Valine	1.75	1.66	1.58
<u>Non-essential amino acids</u>			
Alanine	1.71	1.99	1.87
Aspartic acid	3.4	2.78	2.57
Glycine	2.79	2.55	2.06
Glutamic acid	5.01	4.18	3.95
Proline	2.11	2.67	2.51
Serine	1.67	1.83	1.88
Tyrosine	1.42	1.07	1.07
<b>Fatty acid profile (% FA)</b>			
16:0	22.05	20.82	18.79
18:2 $\omega$ 6	20.49	26.04	35.15
18:3 $\omega$ 3	1.63	1.44	1.62
20:4 $\omega$ 6	0.26	0.25	0.15
20:5 $\omega$ 3	0.03	0.03	0.02
22:6 $\omega$ 3	0.07	0.07	0.04
Total SFA	36.73	33.54	28.21
Total MUFA	38.54	36.49	33.39
Total n-6	21.18	26.63	35.57
Total n-3	1.84	1.62	1.73
Total PUFA	23.02	28.25	37.31

<sup>1</sup> Dried distiller's grain with solubles from a Hungarian company; <sup>2</sup> Hungarian commercial vitamin and mineral premix; SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids.

**Blood plasma clinical chemistry.** The plasma samples were analyzed using an Olympus AU400 automatic biochemical analyzer (Beckman Coulter, USA) at the University of Veterinary Medicine, Budapest, Hungary. Plasma enzymes and metabolites, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), gamma-glutamyltransferase (GGT), lipase, amylase, total cholesterol (TC) and triglyceride (TG) were measured according to IFCC (International Federation of Clinical Chemistry).

**Histology.** For histological analysis, liver and mid-gut samples (n=6 per treatment) were immediately immersed in Bouin's solution for 16 hours and then transferred to 70% ethanol (Culling 1974). Subsequently, the samples were embedded in paraffin and thin sections (5 µm) were obtained and stained with Mayer's hematoxylin and eosin. The morphological structures of these tissues were observed using an imaging microscope (ECLIPSE 80i, Nikon, Japan).

**RNA isolation and RT-PCR.** Expression levels of genes involved in growth (GH), the antioxidant system (SOD-1, GPx) and non-specific immune response (IGF, HSP-70, TNF-α) were measured in liver and head kidney samples, respectively, by real-time quantitative PCR (qPCR), using β-actin as an internal reference gene. Total RNA was isolated from liver and head kidney samples using the SV Total RNA Isolation System (Promega, USA) according to the manufacturer's instructions. The quantity of the RNA was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The integrity (quality) was checked by denaturing gel electrophoresis (1.5% agarose gel) and the purity by measuring the OD260/OD280 absorption ratio (>1.95). The cDNA was generated from 1 µg of total RNA, using the iScript cDNA Synthesis Kit (BioRad) following the manufacturer's protocol. The product of the first-strand cDNA synthesis was stored at -20°C until the quantitative RT-PCR (qRT-PCR) runs. The qPCR reactions were carried out using a LightCycler 96 instrument and the FastStart Essential DNA Green Master qRT-PCR kit (Roche, Switzerland) (primers are presented in Table 2); the solutions consisted of 10 µL master mix (2x), 1 µL PCR forward primer (10 µM), 1 µL PCR reverse primer (10 µM), 5 µL cDNA (reverse transcription reaction mix) and 3 µL nuclease-free water. The thermal profile for all reactions was 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. The specificity of the reactions was checked by melting curve analysis, and no mispriming or primer dimers were found. All reactions were done in triplicates. The mean threshold cycle (Ct) values were calculated and the qPCR data were analyzed by the method described by Pfaffl (2001). Efficiencies of qPCR reactions were determined using standard curves, and serial dilutions were made from cDNAs of a head kidney and a liver sample from both species. These cDNAs were diluted to 10x, 30x, 90x, 270x and 810x. Quantitative PCR reactions were carried out on these dilutions with all four primer pairs in triplicates. Standard curves were drawn for each primer pair by plotting Ct values against the log10 of different dilutions of cDNA sample solutions. Relative mRNA levels were presented as mean values ± S.E.M. of three independent experiments. Efficiencies (E) were calculated from the slopes of the standard curves, applying the equation  $E=10^{(-1/\text{slope})}$ . In *C. carpio* head kidney samples, E values were 2.11 for β-actin, 1.95 for HSP-70 and 2.08 for TNF-α. In *C. carpio* liver samples, E values were 2.11 for β-actin, 1.88 for GH and 2.06 for SOD-1.

Table 2

Oligonucleotide primer sequences

Gene	Primer sequence (5' → 3')	Accession number
GH	TCTTCGCATCTCTTTTACC	FJ265047.1
SOD-1	GACAACACAAACGGCTGCAT	NM_131294
HSP70	TCAGTCTGCCCTTGTCATTGGTGA	AY120894
TNF-α	GCTGTCTGCTTCACGCTCAA	AJ311800
β-actin	AGTTGAGTCGGCGTGAAGTGGTAA	M24113

**Calculations and statistical analysis.** We used the following equations:

$$\begin{aligned}\text{Weight Gain (WG)} &= (\text{final weight} - \text{initial weight}) \times 100 / (\text{initial weight}) \\ \text{Daily Growth Index (DGI)} &= 100 \times [(\text{final weight})^{1/3} - (\text{initial weight})^{1/3}] \times (\text{days}^{-1}) \\ \text{Feed Conversion Ratio (FCR)} &= \text{offered feed (g)} / (\text{final body weight (g)} - \text{initial body weight (g)}) \\ \text{Specific Growth Rate (SGR)} &= (\text{Ln final body mass} - \text{Ln initial body mass}) \times 100 / \text{days} \\ \text{Protein efficiency ratio (PER)} &= \text{weight gain (g)} / \text{protein intake (g)} \\ \text{Protein productivity value (\%)} (\text{PPV}) &= (\text{avg. final weight (g)} \times \text{final whole body crude protein} - \text{avg. initial weight (g)} \times \text{initial whole body crude protein}) / (\text{feed protein} \times \text{FCR} (\text{avg. final weight} - \text{avg. initial weight})) \times 100 \\ \text{Survival rate (SR)} &= [(\text{number of fish at the beginning of the experiment} - \text{mortality}) / \text{number of fish in the beginning of the experiment}] \times 100 \\ \text{Condition factor (CF)} &= \text{body weight (g)} \times 100 / \text{body length}^3 \\ \text{Viscerosomatic index (VSI)} &= \text{total wet viscera (g)} / \text{body weight (g)} \\ \text{Hepatosomatic index (HIS)} &= \text{wet hepatopancreas weight (g)} / \text{wet body weight (g)} \times 100 \\ \text{Visceral fat index (VFI)} &= \text{wet visceral fat (g)} / \text{wet body weight (g)} \times 100 \\ \text{Intestinal somatic index (ISI)} &= \text{wet intestine weight (g)} / \text{wet body weight (g)} \times 100\end{aligned}$$

To compare and evaluate the results, we used SPSS 22.0 for Windows. All data were tested with one-way analysis of variance (ANOVA) with Tukey's Post Hoc test. The statistical IDs marked with different letters translate into a deviation on a significance level of  $p < 0.05$ .

**Results.** After the 12-week trial, statistical differences in terms of growth and feed conversion parameters (Table 3) were observed. *C. carpio* demonstrated significant advantages for groups fed with DDGS-containing experimental feeds compared to the control in WG, DGI, FCR and SGR. Significant differences were not detected between DDGS 20 and DDGS 40, but slightly higher values were measured in DDGS 40 for most of the parameters. Protein efficiency ratios were significantly different between all groups, and the highest nitrogen efficiency use was found in the DDGS 40 group. Previous advantages, like better nitrogen utilization of DDGS groups were strengthened by protein production values. Fish mortality was below 4% in each group. The examined fish did not showed differences in biometric indices, irrespective of the dietary composition (Table 4).

Table 3

Growth performance and feed utilization parameters of *Cyprinus carpio* juveniles

Specification	DDGS 00	DDGS 20	DDGS 40	p-value
IBW (g)	64.46±1.50	60.80±4.04	63.78±2.50	0.477
FBW (g)	186.24±4.02 <sup>a</sup>	202.09±12.88 <sup>ab</sup>	215.06±1.63 <sup>b</sup>	0.012
WG (%)	188.93±10.54 <sup>a</sup>	232.37±16.96 <sup>b</sup>	237.16±13.35 <sup>b</sup>	0.016
DGI (%)	2.07±0.07 <sup>a</sup>	2.36±0.11 <sup>b</sup>	2.43±0.06 <sup>b</sup>	0.006
FCR (g g <sup>-1</sup> )	2.08±0.05 <sup>a</sup>	1.82±0.01 <sup>b</sup>	1.81±0.07 <sup>b</sup>	0.001
SGR (% day <sup>-1</sup> )	1.31±0.04 <sup>a</sup>	1.46±0.06 <sup>b</sup>	1.48±0.05 <sup>b</sup>	0.014
PER (g g <sup>-1</sup> )	1.30±0.04 <sup>a</sup>	1.59±0.01 <sup>b</sup>	1.68±0.07 <sup>c</sup>	<0.001
PPV (%)	18.79±0.55 <sup>a</sup>	23.10±0.24 <sup>b</sup>	23.98±0.96 <sup>b</sup>	<0.001
SR (%)	96.67	100.00	98.89	-

IBW: initial body weight; FBW: final body weight; WG: weight gain; DGI: daily growth index; FCR: feed conversion ratio; SGR: specific growth rate; PER: protein efficiency ratio; SR: survival rate. Values are means of three replicates; values within the same row with different letters are significantly different ( $p < 0.05$ ). Data are presented as mean ±SD.

Table 4

## Biometric indices at the end of the experiment

Specification	DDGS 00	DDGS 20	DDGS 40	p-value
CF (g cm <sup>-3</sup> )	1.60±0.31	1.54±0.12	1.49±0.16	0.51
VSI (%)	11.42±2.03	12.1±2.04	11.14±1.52	0.63
HSI (%)	2.25±0.34	2.53±0.12	2.46±0.20	0.15
VFI (%)	0.77±0.45	0.33±0.12	0.56±0.23	0.06
ISI (%)	3.05±0.31	3.33±0.29	3.36±0.35	0.22

CF: condition factor; VSI: viscerosomatic index; HSI: hepatosomatic index; VFI: visceral fat index; ISI: intestinal somatic index. Values are means of six replicates; values within the same row with different letters are significantly different ( $P < 0.05$ ). Data are presented as mean ±SD.

The results of whole-body composition are shown in Table 5. Crude protein content was significantly higher ( $p < 0.05$ ) in the DDGS fed groups compared to the zero level. Parallel with this, crude fat content increased, while crude ash level decreased with higher DDGS inclusion levels.

Table 5

## Whole body proximate compositions (for dry matter)

Specification	DDGS 00	DDGS 20	DDGS 40	p-value
Crude protein (%)	52.37±1.04 <sup>a</sup>	55.84±1.38 <sup>b</sup>	56.28±1.96 <sup>b</sup>	0.044
Crude fat (%)	38.52±0.94 <sup>a</sup>	34.79±1.10 <sup>b</sup>	34.12±2.08 <sup>b</sup>	0.024
Crude ash (%)	6.29±0.42 <sup>a</sup>	6.83±0.23 <sup>b</sup>	7.74±0.45 <sup>b</sup>	0.009

Values are means of six replicates; values within the same row with different letters are significantly different ( $P < 0.05$ ). Data are presented as mean ±SD.

The results of plasma biochemical parameters showed no differences between the experimental groups (Table 6).

Regarding the fatty acid composition of the liver, the level of linoleic acid (18:2 n-6) in the tissues reflects the dietary trends. The synthesis of a higher homologue such as arachidonic acid was detectable. The EPA and DHA levels were extremely low in the liver. Statistically significant differences were found for oleic acid (18:1n-9), and the highest deposition was observed in the DDGS 00 group (Table 7), where more than 40% of the lipid were composed from this fatty acid. A similar tendency could be observed for the total MUFA level, which decreased with an increasing inclusion level of DDGS in the diet, while the total PUFA level increased. The lipid content of the liver was 65.19 mg g<sup>-1</sup>. Total n-3/total n-6 ratio was very low. The EPA + DHA levels ranged between 1.40 and 1.78% in *C. carpio* and 0.37%.

Table 6

## Serum biochemical parameters at the end of the experiment

Parameter	Unit	DDGS 00	DDGS 20	DDGS 40	p-value
TC	mmol/L	4.23±0.71	4.30±0.32	4.00±0.40	0.572
TG	mmol/L	5.03±1.36	3.66±0.82	4.13±0.32	0.065
ALT	U/L	3.33±1.03	3.00±1.09	3.33±1.03	0.821
AST	U/L	136.3±49.6	137.7±47.9	180.0±155.2	0.687
AP	U/L	112.0±29.9	124.8±17.6	85.6±27.9	0.087
GGT	U/L	2.42±0.19	2.48±0.24	2.46±0.27	0.943
Amylase	U/L	106.4±50.9	114.3±56.2	98.3±19.5	0.828
Lipase	U/L	14.33±2.33	14.00±1.79	16.00±1.79	0.209

TC: total cholesterol; TG: triglyceride; ALT: alanine aminotransferase; AST: aspartate aminotransferase; AP: alkaline phosphatase; GGT: gamma-glutamyltransferase. Values are means of six replicates; values within the same row with different letters are significantly different ( $P < 0.05$ ). Data are presented as mean ±SD.

Histological analysis in the liver of *C. carpio* (Figure 1) showed that the group fed with 40% DDGS had hepatocellular necrosis, tight sinusoids and hypertrophia. The experimental diets did not affect gut health, and only generic differences (thickness of epithelium, size and number of goblet cells) were found in histological sections. Based on the results of the RT-PCR, relative expression levels of GH, SOD-1 and GPx genes in the liver or IGF, HSP-70 and TNF- $\alpha$  genes in the head kidney showed no significant differences between the groups DDGS 20 and DDGS 40 and the control group (Figures 2 & 3).

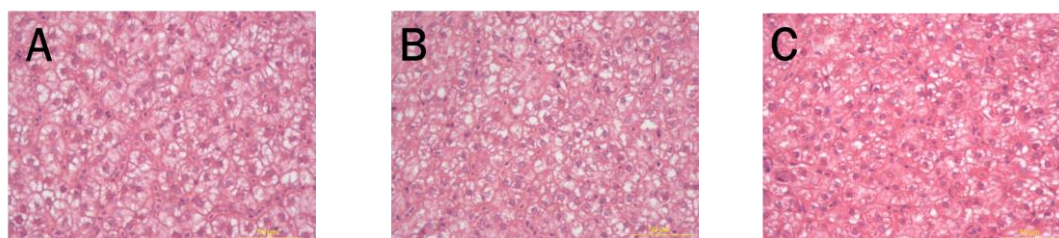


Figure 1. Hepatocyte construction of *Cyprinus carpio*. A = DDGS 00; B = DDGS 20; C = DDGS 40.

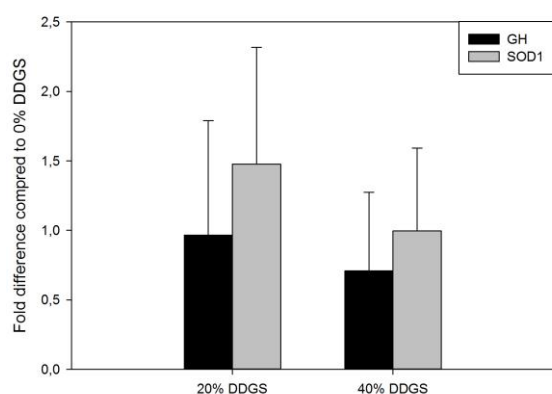


Figure 2. Expression of growth hormone (GH) and superoxid dismutase 1 (SOD1) genes in liver.

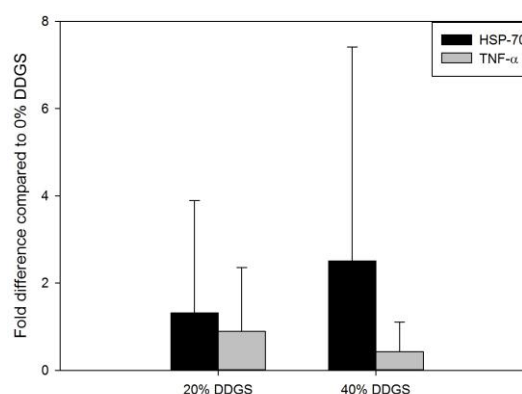


Figure 3. Expression of heat shock protein 70 (HSP-70) and tumour necrosis factor -  $\alpha$  (TNF- $\alpha$ ) genes in head kidney.

Table 7

Fatty acid composition of *Cyprinus carpio* liver

Fatty acid	DDGS 00	DDGS 20	DDGS 40	p-value
w% FA				
16:1 $\omega$ 9	0.70 $\pm$ 0.11	0.69 $\pm$ 0.08	0.62 $\pm$ 0.06	0.244
16:1 $\omega$ 7	4.10 $\pm$ 0.37 <sup>b</sup>	3.77 $\pm$ 0.72 <sup>ab</sup>	3.13 $\pm$ 0.43 <sup>a</sup>	0.019
18:1 $\omega$ 9	41.16 $\pm$ 1.73 <sup>c</sup>	37.18 $\pm$ 2.70 <sup>b</sup>	32.67 $\pm$ 1.32 <sup>a</sup>	<0.001
18:1 $\omega$ 7	2.76 $\pm$ 0.12 <sup>b</sup>	2.58 $\pm$ 0.23 <sup>b</sup>	2.22 $\pm$ 0.09 <sup>a</sup>	<0.001
18:2 $\omega$ 6	10.75 $\pm$ 0.81 <sup>b</sup>	13.45 $\pm$ 2.61 <sup>b</sup>	16.61 $\pm$ 2.02 <sup>a</sup>	<0.001
18:3 $\omega$ 3	0.61 $\pm$ 0.06	0.56 $\pm$ 0.12	0.60 $\pm$ 0.06	0.452
20:3 $\omega$ 9	0.73 $\pm$ 0.16	0.66 $\pm$ 0.20	0.64 $\pm$ 0.13	0.594
20:4 $\omega$ 6	4.50 $\pm$ 0.68	4.43 $\pm$ 1.86	6.21 $\pm$ 1.44	0.077
20:5 $\omega$ 3	0.06 $\pm$ 0.01 <sup>b</sup>	0.07 $\pm$ 0.01 <sup>b</sup>	0.08 $\pm$ 0.02 <sup>a</sup>	0.07
22:6 $\omega$ 3	1.45 $\pm$ 0.36	1.32 $\pm$ 0.62	1.69 $\pm$ 0.36	0.394
Total lipid mg/g sample	96.13 $\pm$ 18.97 <sup>b</sup>	86.88 $\pm$ 28.21 <sup>b</sup>	65.19 $\pm$ 13.62 <sup>a</sup>	0.076
Total SFA	24.34 $\pm$ 0.59 <sup>b</sup>	25.72 $\pm$ 0.61 <sup>b</sup>	24.49 $\pm$ 0.82 <sup>a</sup>	0.006
Total MUFA	51.66 $\pm$ 1.72 <sup>c</sup>	47.04 $\pm$ 3.41 <sup>b</sup>	41.24 $\pm$ 1.52 <sup>a</sup>	<0.001
Total PUFA	21.39 $\pm$ 1.35 <sup>b</sup>	24.62 $\pm$ 3.28 <sup>b</sup>	31.88 $\pm$ 2.02 <sup>a</sup>	<0.001
Total n-3/Total n-6	0.12 $\pm$ 0.01 <sup>b</sup>	0.09 $\pm$ 0.02 <sup>a</sup>	0.09 $\pm$ 0.01 <sup>a</sup>	0.002

Values are means of six replicates; values within the same row with different letters are significantly different (P<0.05). Data are presented as mean  $\pm$ SD. SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids.



**Discussion.** The results of the current study show that replacement of dietary soybean meal and corn grain with a by-product of the bioethanol industry, namely DDGS, had positive effects on the growth performance of *C. carpio*. Similar tendencies have been observed for *I. punctatus* (Li et al 2011b), *O. mykiss* (Øverland et al 2013) and *O. niloticus* (Wu et al 1996, 1997). However, another study with *O. niloticus* showed reduced growth performance as DDGS level increased to up to 20% (Khalil & El-sharkawy 2013). For *C. carpio*, feed utilization of DDGS-containing diets was better compared to the diets with high levels of soybean and corn grain. This confirms that DDGS is an easily digestible and usable feedstuff for *C. carpio*. The protein efficiency ratio and protein productivity values determined using DDGS 40 were comparable with results coming from studies with other plant protein sources (Hasan et al 1997) and are relatively good for *C. carpio*.

Feeds containing DDGS had beneficial effects on the body composition by reducing the fat and increasing the protein contents. However, Khalil & El-sharkawy's (2013) found opposite effects with *O. niloticus*. Furthermore, the decreased body fat content is correlated with the blood plasma's TC and TG values, which showed the same pattern. The reduced HSI and VSI values in groups fed with DDGS correlated with the salutary effects on the whole-body compositions due to the DDGS content in the feeds. Schaeffer et al (2009) found a similar decrease in HSI values with *O. niloticus* fed with 40% DDGS.

We found no histological differences - such as enteritis features, the size of the goblet cells, epithelium thickness - in the intestines between the experimental groups, in contrast to the effects observed for soybean meal (Urán et al 2008). Markovic et al (2012) reported that the optimal yeast: soybean meal ratio can reduce enteritis symptoms with slight histological changes. The liver histopathology of farmed fish fed artificial diets often shows lipid accumulation, vacuolic degeneration, hypertrophy and hepatic cell degeneration (Coz-Rakovac et al 2005; Bilen & Bilen 2013). In this study, such changes were also observed. Hepatocellular necrosis was only found in few samples and can therefore not be used as a characterization. Caballero et al (2002), Pereira et al (2002), Figuerado-Silva et al (2005) and Fountoulaki et al (2009) also found no liver histological changes between the experimental groups when they changed the diet's fish oil to vegetable oil for *O. mykiss*, *D. labrax* and gilthead seabream (*Sparus aurata*). Unfortunately, plant protein sources contain numerous different anti-nutrient factors (ANFs). If these ingredients are supplemented in the diet for fish, they can influence fish health (Francis et al 2001). It is considered that DDGS - in the absence of ANFs - is not responsible for the observed histological changes.

The lack of plasma biochemical reference data makes it difficult to compare the results with other studies (Coz-Rakovac et al 2005); in addition, such findings are also a factor of fish size, species, environmental circumstances (Bowser 1993; Chen et al 2003). The parameters ALT, AST, AP and GGT are important indicators reflecting liver injury. In this study, for *C. carpio*, plasma biochemical parameters showed no significant differences between the experimental groups. Plasma GGT values were below 2.5 U/L, which is normal for untreated *C. carpio* according to Velisek et al (2009) and for *O. niloticus* according to Chen et al (2003). In this study, the mean values of AST and AP were not significantly different because of the relatively high standard deviation. While the ALT values were lower, AST and AP were slightly higher than in the study by Wang et al (2014), who substituted soybean meal with cottonseed meal in the diets of *C. carpio*. In contrast to fish meal substitutional studies with plant sources, Glencross et al (2011) reported the same low values of ALT in fish meal diets, albeit with elevated ALP values in barramundi (*Lates calcarifer*). The author also suggests that the activity of ALT is the main indicator of liver damage; plasma ALT levels were relatively low, and ALP levels did not significantly differ between groups, indicating that DDGS does not cause liver damage in *C. carpio*. As a vital energy source of cells, the levels of TG and TC, important cell membrane components and precursors of steroid hormones, indicate the nutritional condition of fish (Groff & Zinkl 1999). In *C. carpio*, we found no significant differences in TG and TC values.

The higher levels of amylase and lipase in the blood plasma indicated acute pancreatitis, although these values are variable and often depend on the feeding habit of

fish (Thrall et al 2012). In this study, we found no differences between the experimental groups in terms of lipase values. There were no differences in plasma amylase concentrations, too.

Diet is the most important factor that influences the fatty acid composition of the body (Miles & Chapman 2015). The increase in total PUFA due to the addition of corn DDGS, as well as the increase in saturated FA due to the animal by-product used in the diet, was expected. The high endogenous production of 20:4n-6 suggests the availability of 18:2n-6 in the diet and powerful retention, especially in the case of DDGS 40 group. The extremely low amount of n-3 in the diet contributed to a low level and a low n-3/n-6 ratio in the liver, with a low sum of EPA and DHA. MEAD acid, 20:3n-9, as an indicator of essential fatty acid deficiency, could be detected (Takeuchi & Watanabe 1977). High amounts of MUFA (18:1n-9 and 16:1n-7) indicate liver fattening (Torstensen et al 2011), which could be seen even in the total lipid level of the samples. Total lipid amounts (mg g<sup>-1</sup>) differed significantly between DDGS 40 and DDGS20, and DDGS 40 and DDGS 00 groups. The highest fat deposition was observed in the DDGS 00 group.

Genes related to growth (GH and IGF), antioxidant status (SOD1, GPx), inflammatory genes (TNF- $\alpha$ ) and stress (HSP70) related genes were not affected by the experimental feeds. Urán et al (2008), and Zhou et al (2016) demonstrated that the substitution of fish meal by a mixture of soybean meal and fermented meal resulted TNF- $\alpha$  gene upregulation. Hemre et al (2004) observed HSP70 gene expression when fish were fed with suboptimal diets (Zhang et al 2009). In a similar experiment, when Zheng et al (2012) replaced soybean meal with cottonseed meal in the diet of grass carp (*Ctenopharyngodon Idella*), antioxidant enzyme genes were upregulated with increasing replacement levels. However, in our study, gene expression did not differ between the treatments with different dietary levels of DDGS.

**Conclusions.** The results of the present study indicate that DDGS at high inclusion levels (up to 40%) represents an appropriate dietary protein and fat source for *C. carpio*. The study demonstrated that the decrement of liver fat deposition is remarkable after a three-months feeding period using diet with high percentage (40%) of DDGS. Blood plasma biochemical tests could successfully be used to monitor the metabolic balance and health status of fish. In our study, these parameters correlated with the histological results.

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