Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X 2019 / v.21 / n.3 / 001-008

http://dx.doi.org/10.1590/1806-9061-2019-1025

Original Article

Apparent Faecal Digestibility of Essential Amino Acids from Ω3 Pufa Diets for Laying Hens

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■Keywords

Amino acid digestibility, PUFA enriched diets, laying hen.

Submitted: 20/February/2019
Approved: 14/June/2019

ABSTRACT

The apparent faecal digestibility of the essential amino acids from Ω3 PUFA layer diets and feeding solutions to avoid possible adverse effects of such diets were investigated in two experiments conducted on Lohmann Brown laying hens. Experiment 1 used 72 layers assigned to three groups (C1, E1-1, E2), while experiment 2 used 96 layers assigned to four groups (C2, E1-2, E3, E4). All experimental diets have been enriched in Ω 3 PUFA by supplementing a conventional diet (C1, C2) with 5% flaxseed meal and 2% camelina meal. Diets E2 and E4 had an increased level of Cu (150 mg/kg), diets E3 and E4 were supplemented with 0.0125% enzyme mix, while diets E1-1 and E1-2 were only supplemented with 5% flaxseed meal and 2% camelina meal. The digestibility coefficients of the studied essential amino acids (threonine, valine, phenylalanine, isoleucine, leucine, lysine, cystine, methionine) were lower ($p \leq 0.05$) in groups E1-1 and E2 from experiment 1, than in the control group (C1). In experiment 2, the supplemental enzyme mix (E3) increased ($p \leq 0.05$) the digestibility coefficients of phenylalanine, lysine, cystine and methionine, compared to the diet with camelina and flaxseed meals (E1-2). The simultaneous inclusion of copper and enzyme mix in the E4 diet increased ($p \leq 0.05$) lysine digestibility compared to group E1-2 and sulphur amino acids digestibility, compared to the other groups (C2, E1-2 and E3). In both experiments, only the feed conversion ratio was different ($p \leq 0.05$) in favour of the control groups (C1 and C2), compared to the other groups.

INTRODUCTION

A major interest to increase the concentration of Ω-3 polyunsaturated fatty acids (Ω-3 PUFA) in human diets was noticed over the past two decades, following the research studies which proved their beneficial effects for human health (Liu & Kim, 2018; Shahidi & Ambigaipalan, 2018). Since the feeding value of the egg can be influenced by nutrition, Ω-3 PUFA content from the hen's egg yolk can be increased in the same manner. Flaxseeds (*Linum usitatissimum)* and camelina (*Camelina sativa*) are among the oleaginous crops successfully used in layer diets to enrich the eggs in Ω-3 PUFA (Cherian, 2017; Olteanu *et al.*, 2016, Criste *et al.*, 2009, Zotte *et al.*, 2015), but the dietary level is limited by their antinutritional factors (Woyengo *et al.*, 2017, Pekel *et al.*, 2015).

The Cu-sulfate supplementation may redirect glucosinolates breakdown products, or produce secondary breakdown products by rearrangement reactions (Tripathi & Mishra, 2007). Thus, dietary Cu supplementation can influence in a positive manner the nutritive value and potential toxic effects of the camelina meal. Pekel *et al.* (2009) noticed that Cu supplementation at the prophylactic level (150 mg/

kg) indicated an ability to improved live performance and carcass parameters of broilers fed the camelina diet. Youvalari *et al.* (2017) reported that treatments of canola meal, which is a member of the Brassica family with Cu-sulfate could alleviate adverse effects of glucosinolate on broilers performance.

Another method of alleviating the effects of the antinutritional factors, mentioned in literature, is the use of enzymes, like xylanase, amylase, protease, and phytase, in the corn-soybean diets for layers, which enhances layer performance (Cowieson & Adeola, 2005) and which increases the apparent metabolisable energy (Meng *et al.*, 2005) and the ileal digestibility of protein and amino acids (Zanella *et al.*, 1999).

Limited information on amino acid digestibility from camelina meal and flaxseed meal for poultry are available. A few studies reported that camelina cake can be considered an energy source given its remaining oil content, but its digestibility coefficients for amino acid and crude protein are low indicating that it may contained high glucosinolate concentrations generally observed in camelina meal, thus limiting its utilization in swine diets (Kahindi *et al.*, 2014; Graham *et al.*, 2013).

The purpose of this study was to investigate in two *in vivo* digestibility trials on layers, the effects of Ω 3 PUFA-enriched diet on the apparent faecal digestibility of the essential amino acids, as well as various feeding solutions to control the possible adverse effects that may occur in such situations.

MATERIAL AND METHODS

All animal experiments were conducted in compliance with the European Union Directive 2010/63/EU and in accordance with regulations set by the Ethical Commission of National Research and Development Institute for Biology and Animal Nutrition.

Experimental design

Experiment 1. The trial was conducted for 6 weeks on 72 Lohmann Brown layers (60 weeks), assigned to three groups (C1, E1-1, E2). Compared to the control diet (C1), the diet formulations for the experimental groups (E1-1 and E2) included 5% flaxseed meal and 2% camelina meal (Table 1). Compared to 6 mg Cu/kg feed from the premix of C1 and E1-1 diets, E2 diet had 150 mg Cu/kg feed (from $CuSO_4 \cdot 5H_2O$).

Experiment 2. The trial was conducted for 4 weeks on 96 Lohmann Brown layers (58 weeks) assigned to four groups (C2, E1-2, E3, E4). Compared to the control diet (C2), similar to C1 from experiment 1, the diet formulations for the experimental groups (E1-2, E3 and E4) included 5% flaxseed meal and 2% camelina meal (Table 1). Compared to E1-2 diet (similar to E1-1), E3 and E4 diets contained 0.0125 kg cellulolytic enzyme/100 kg feed (Table 1). The enzyme was Biozim M6000 (Biomin, Austria), with β-xylanase (6000 U/g) as active substance, and β-glucanase as secondary substance. Compared to E3 diet, E4 diet had 150 mg Cu/kg feed, from $CuSO_4 \cdot 5H_2O$.

The hens used in both trials have been housed in experimental halls fitted with digestibility cages (2 layers per cage), which allow accurate weighing of the daily ingesta and excreta, with controlled environmental conditions (average temperature 23±1°C; humidity 65±3%; light regimen 6 h/24h). Feed and water were supplied *ad libitum*. The productive parameters (feed conversion, laying percentage, average egg weight) were calculated from the daily records.

Balance period. The apparent faecal amino acids digestibility was evaluated using the methodology of Sirirat *et al.* (2013). The balance was conducted for 5 days, during week 3. During this period, feed intake and average weights from each two cages (6 samples/group) of excreta voided (dry matter basis) were recorded and sampled for amino acid analysis. Amino acid retention ratio was calculated using the following equation:

$$\text{Amino acid retention ratio (\%)} = \frac{(\text{WFI x AAF}) - (\text{WEV x AAE})}{(\text{WFI x AAF})} \text{ x } 100$$

where: WFI = weight of feed intake; AAF = concentration of amino acid in feed; WEV = weight of total excreta voided; AAE = concentration of amino acid in total excreta.

Chemical analysis

Proximate composition. Standard methods were used to determine the concentration of crude protein (Kjeltec auto 1030 – Tecator Instruments, Hoganas, Sweden), crude fat (Soxtec 2055 – Foss Tecator, Sweden), crude fibre (Fibertec 2010 System – Foss Tecator, Sweden), crude ash (Caloris CL 1206 furnace, Romania) from feeds according to Regulation (EC) no. 152/2009. The fatty acids were determined by the gas chromatography method (AOAC, 2000) using a Perkin-Elmer Clarus 500 GC (Massachusetts, United States), fitted with Flame Ionization Detector (FID) and capillary separation column with high polar stationary phase TRACE TR-Fame, (Thermo Electron, Massachusetts, United States), size 60 m X 0.25 mm X 0.25 μm.

Amino acids determination from samples of ingesta and droppings was performed by HPLC, using a method optimised and validated by Varzaru *et al.* (2013), and HPLC system Finnigan Surveyor Plus and HyperSil BDS C18 column, size 250 × 4.6 mm, 5μm (Thermo-Electron Corporation, Waltham, MA).

Statistical analysis

The statistical analyses were performed using STATVIEW software for Windows (SAS, version 6.0). The data were subjected to one-way ANOVA followed by Fisher's test to detect the differences between the groups. Values of *p* less than 0.05 were considered significantly different.

Table 1 – Formulations of the compound feeds used in the two trials.

Item	Experiment 1			Experiment 2			
	C1	E1-1	E2	C2	E1-2	E3	E4
Corn, %	35.74	33.75	33.75	35.74	33.75	33.74	33.74
Rice, %	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Wheat, %	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Rapeseed meal, %	15.00	9.50	9.50	15.00	9.50	9.50	9.50
Soybean meal, %	9.00	9.00	9.00	9.00	9.00	9.00	9.00
Gluten, %	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Flaxseed meal,%	-	5.00	5.00	-	5.00	5.00	5.00
Camelina meal, %	-	2.00	2.00	-	2.00	2.00	2.00
Oil, %	2.00	2.40	2.40	2.00	2.40	2.40	2.40
Monocalcium phosphate, %	1.06	1.06	1.06	1.06	1.06	1.06	1.06
Calcium carbonate, %	8.70	8.70	8.70	8.70	8.70	8.70	8.70
Salt, %	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Methionine, %	0.15	0.12	0.12	0.15	0.12	0.12	0.12
Lysine, %	-	0.12	0.12	0.12	0.12	0.12	0.12
Choline, %	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Enzyme, %	-	-	-	-	-	0.0125	0.0125
Vitamin-mineral premix, %	1.00*	1.00*	1.00**	1.00*	1.00*	1.00*	1.00**
Total	100	100	100	100	100	100	100

*1 kg premix contains: 1350000 IU/kg vit.A; 300000 IU/kg vit.D3; 2700 IU/kg vit.E; 200 mg/kg Vit.K; 200 mg/kg Vit.B1; 480 mg/kg Vit.B2; 1485 mg/kg pantothenic acid; 2700 mg/kg nicotinic acid; 300 mg/kg vitamin B6; 4 mg/kg vitamin B7; 100 mg/kg vitamin B9; 1.8 mg/kg vitamin B12; 2500 mg/kg vitamin C; 7190 mg/kg manganese; 6000 mg/kg iron; 600 mg/kg copper; 6000 mg/kg zinc; 50 mg/kg cobalt; 114 mg/kg iodine; 18 mg/kg selenium; ** The premix contains 15000 mg Cu/kg compound feed.

RESULTS

Feeding quality of the feeds

The tested compound feeds formulations were isocaloric and isoprotein (Table 2). The small differences in the proximate chemical composition and in the amino acid's concentrations in the feeds from the two trials were due to the variation in the chemical composition of the raw materials used to manufacture the compound feeds, and also to the

Table 2 – Data on nutrients content of compound feeds used in the two trials

Item	Experiment 1			Experiment 2			
	C1	E1-1	E2	C2	E1-2	E3	E4
ME, Kcal/kg	2648.69	2665.43	2665.4	2540.8	2678.62	2678.62	2678.62
Protein, %	17.01	17.77	17.72	16.77	16.06	16.29	16.77
Fat, %	6.00	7.20	6.98	6.10	7.43	7.62	7.14
PUFA omega 3	1.96	8.51	8.28	1.82	8.55	8.14	8.17
Fibre, %	6.01	6.64	6.71	5.24	5.79	5.38	5.21
Ash, %	13.29	11.99	12.38	13.35	11.28	12.25	11.40
Cu, mg/kg	6.28	6.70	156.33	11.11	11.72	15.84	161.05
Essential amino acids							
Threonine, %	0.65	0.61	0.63	0.71	0.70	0.67	0.67
Valine, %	0.98	0.93	0.92	1.08	1.11	1.04	1.02
Phenylalanine, %	0.72	0.69	0.68	0.77	0.80	0.75	0.78
Isoleucine, %	0.61	0.57	0.55	0.68	0.71	0.66	0.70
Leucine, %	1.34	1.25	1.27	1.47	1.47	1.41	1.44
Lysine, %	0.76	0.80	0.77	0.76	0.73	0.73	0.79
Cystine, %	0.27	0.28	0.27	0.32	0.28	0.31	0.32
Methionine, %	0.45	0.42	0.43	0.38	0.36	0.37	0.37

uncertainty degree of the analytical methods used for the respective determinations. The use of flaxseed and camelina meals in the formulations for the experimental groups from the two experiments, led to enriched diets in α linolenic acid (PUFA Ω 3 acid), in average by 5.2 times compared to C1 and C2. In order to control the adverse effects of the antinutritional factors present in the two oleaginous meals, E2 and E4 diets, both enriched in PUFA Ω 3, have been supplemented with copper, compared to C1 and C2, respectively, as shown by their concentration of this trace element. Moreover, E4 diet was also supplemented with exogenous enzymes.

Layer performance

Experiment 1. The results obtained for feed conversion ratio (Table 3) were better ($p \leq 0.05$) in the control group (C1) compared to the experimental groups enriched in PUFA Ω3, but the laying percentage and the average egg weight were not different ($p > 0.05$) between the groups.

Experiment 2. Table 3 data shows a better feed conversion ratio ($p \leq 0.05$) in group E4 (enriched in PUFA Ω3, supplemented with Cu and enzyme mix), compared to E1-2 (enriched in PUFA Ω3), to E3 (enriched in PUFA Ω3 and enzyme mix) and to C2. The laying percentage was higher ($p > 0.05$) in E4 than in E1-2. The average egg weight didn't vary significantly in the groups (E3 and E4) compared to C2.

Apparent faecal digestibility of the essential amino acids

Experiment 1. In experiment 1, the digestibility coefficients of the groups enriched in PUFA Ω 3 (E1-1 and E2), were lower ($p \leq 0.05$) than in the control group (C1), for the studied essential amino acids (Table 4).

The results showed that in E1-1 diet (rich in PUFA Ω 3 but with no supplemental Cu), lysine digestibility decreased with 2.1%, and methionine digestibility decreased with 3.3% compared to C1. Also, cystine digestibility decreased with 2.1% in E1-1 and with 2.06% in E2, compared to C1. The higher level of Cu from E2 diet increased slightly lysine digestibility (0.08%) and methionine digestibility (1.2%) compared to group E1-1.

Table 3 – Layer performance (average values/group/ experiment).

Item	Experiment 1					Experiment 2					
	C1	E1-1	E2	SEM	P	C2	E1-2	E3	E4	SEM	*p*
Feed conversion ratio (kg CF/kg egg)	1.84 [a]	1.96 [b]	2.02 [b]	0.02	0.01	1.93 [a]	2.01 [ab]	2.04 [b]	1.91 [c]	0.02	0.0382
Laying percentage (%)	87.18 [a]	84.83 [a]	85.06 [a]	0.01	0.3775	88.66 [a]	86.19 [a]	87.32 [a]	90.73 [a]	0.51	0.4073
Average egg weight (g)	64.54 [a]	64.92 [a]	63.28 [a]	0.45	0.2793	65.07 [a]	64.76 [a]	64.94 [a]	65.02 [a]	0.16	0.9165

In experiment 1: means in the same row with no common superscript are significantly different ($p < 0.05$). In experiment 2: means in the same row with no common superscript are significantly different ($p < 0.05$).

SEM: standard error of the mean.

Table 4 – Apparent faecal essential amino acids digestibility (%).

Amino acid	Experiment 1					Experiment 2					
	C1	E1-1	E2	SEM	*p*	C2	E1-2	E3	E4	SEM	*p*
Threonine, %	80.46 [b]	74.29 [a]	74.44 [a]	0.91	0.0007	77.44 [a]	75.65 [a]	76.11 [a]	77.15 [a]	0.37	0.2785
Valine, %	84.30 [b]	78.60 [a]	79.97 [a]	0.86	0.046	80.67 [a]	79.54 [a]	79.54 [a]	81.73 [a]	0.41	0.7585
Phenylalanine, %	88.39 [b]	82.32 [a]	78.77 [c]	1.08	<0.0001	85.52 [c]	81.25 [a]	84.83 [b]	87.21 [d]	0.55	<0.0001
Isoleucine, %	83.20 [b]	76.05 [a]	77.34 [a]	1.00	0.0018	80.21 [ab]	77.97 [a]	80.45 [ab]	81.73 [b]	0.50	0.0384
Leucine, %	88.87 [b]	84.74 [a]	83.86 [a]	0.69	0.0005	86.26 [b]	84.21 [a]	85.64 [ab]	86.07 [ab]	0.34	0.1287
Lysine, %	86.26 [b]	84.41[a]	84.48 [a]	0.37	0.0640	90.21 [b]	88.25 [a]	90.17 [b]	90.48 [b]	0.27	0.0017
Cystine, %	83.79 [b]	81.14 [ab]	80.90 [a]	0.58	0.0796	83.64 [b]	81.64 [a]	84.02 [b]	85.656 [c]	0.39	<0.0001
Methionine, %	89.83 [b]	86.80 [a]	87.91 [a]	0.42	0.0022	86.16 [b]	82.67 [a]	87.43 [b]	90.24 [c]	0.63	<0.0001

In experiment 1: means in the same row with no common superscript are significantly different ($p < 0.05$). In experiment 2: means in the same row with no common superscript are significantly different ($p < 0.05$).

SEM: standard error of the mean.

Experiment 2. Similar to results of experiment 1 for group E1-1, the amino acids balance data (Table 4) showed a lower digestibility of the amino acids for group E1-2, compared to group C2. The enzyme inclusion in E1-2 diet, which thus became diet E3, increased significantly ($p \leq 0.05$) the apparent digestibility coefficients for phenylalanine, lysine, cystine and methionine compared to E1-2. Table 4 also shows that the simultaneous inclusion of copper and enzyme in the diet of group E4 increased ($p \leq 0.05$) lysine digestibility compared to group E1-2; increased ($p \leq 0.05$) sulphur amino acids digestibility compared to groups C2, E1-2 and E3. Compared to group C2, which received a conventional feed, the highest increase of the digestibility coefficients was noticed in group E4, which were with 2.4 % higher for cystine, with 4.7 % for methionine and with 0.3% for lysine. Compared to E1-2 diet (enriched in PUFA Ω3), the use of the enzyme mix (β-xylanase and β-glucanase) and of copper in E4 diet (enriched in PUFA Ω3), increased cystine digestibility with 4.9 %, methionine digestibility with 9.1% and lysine digestibility with 2.5%.

DISCUSSION

Flaxseed and camelina are gaining popularity as feed ingredients because of their high content of PUFA Ω3, and their ability to increase PUFA Ω3 concentration in eggs when included in laying hens diets (Aziza *et al.*, 2013). However, the content of antinutritional factors in camelina and flaxseed meal can decrease the digestibility of dietary proteins. In experiments conducted with broiler chickens, several authors reported significant decreases in digestibility of dry matter, nitrogen, and energy with increasing dietary camelina meal concentration (Acamovic *et al.*, 1999; Thacker & Widyaratne, 2012). Pekel *et al.* (2015) showed a poor energy and nitrogen utilization when camelina meal was added to broilers diet, due to a high viscosity observed in jejunal digesta caused by the glucosinolate content of camelina meal.

Layer performance

The level of 7 % oleaginous meals used in the two experiments was moderate, compared to 10-15 % used in other studies (Cherian *et al.*, 2009; Aziza *et al.*, 2013), and had no significant effect ($p>0.05$) on laying percentage and egg weight. Different results were obtained by Aziza *et al.* (2013) when feeding 10 % flax or camelina meal. The authors observed higher egg production ($p<0.05$) compared with a corn-soy-based control diet. Cherian *et al.* (2009) showed that 15% dietary camelina meal had an adverse effect on the laying percentage and egg weight, compared to the control, while feeding 10% camelina meal only affected egg weight.

The copper supplementation of a diet which includes feeds from Brassica family has been proposed by other authors too (Tripathi & Mishra, 2007; Pekel *et al.*, 2009). On the other hand, Pekel & Alp (2011), who used 250 mg Cu/kg in layer diets with 10% sunflower meal, concluded that increasing the dietary Cu intake did not affect layer performance. Balevi & Coskun (2004) didn't notice significant effects of Cu supplements on egg production, average daily feed intake and feed conversion ratio. Jegede *et al.* (2015) showed that dietary supplements of 50 and 100 mg Cu/ kg feed increased egg production compared to dietary supplements of 150 mg Cu/ kg feed.

The use of exogenous enzymes in cereal-based layer diets is beneficial because they can hydrolyse the non-starch polysaccharides, reduce digesta viscosity and improve nutrient absorption and layer performance (Wang *et al.*, 2005). Unlike other studies (Mirzaie *et al.*, 2012), the average egg weight didn't vary significantly in the groups supplemented with enzyme (E3 and E4) compared to C2. The inclusion of xylanase by Mirzaie *et al.* (2012) increased egg production ($p<0.05$), egg weight ($p<0.01$) and feed conversion ratio ($p<0.01$).

Apparent faecal digestibility of the essential amino acids

Varzaru *et al.* (2015) used 5% flaxseed meal and 2% camelina meal in layer diets, and also noticed a decrease ($p \leq 0.05$) of some amino acids (lysine, methionine, valine, arginine and alanine) versus a corn-soybean meal control. Aziza *et al.* (2013) conducted a trial on layers to evaluate, among others, crude protein digestibility when feeding a corn-soybean diet containing 10% camelina or flax meal and noticed a significant ($p<0.05$) decrease of crude protein digestibility compared with the control diet. Literature has poor data on the effect of the dietary copper on amino acids digestibility. Thus, Rowan *et al.* (1991) unlike the findings of this study, noticed a better digestibility of some amino acids when 200 mg Cu/kg of feed were used in pig diets which included rapeseeds with less than 5 μmol/kg glucosinolates.

Glucosinolates and trypsin inhibitor are the major antinutritional factors in camelina co-products, in which total glucosinolate content ranged from 34.4 to 36.3 μmol/g and trypsin inhibitor activity from 12 to 28 TIU/mg, according to Woyengo *et al.* (2017). In the same study, a content of 127 mg cyanogenic glucoside/ kg from flaxseed meal is reported, cyanogenic glucosides being the major antinutritional factors in flaxseed co-products. The mechanism by which dietary Cu supplementation could overcome the negative effects of glucosinolates can be explained in part by reducing their ileal concentration, by absorption from the intestinal lumen or conversion to other by-products (Tripathi & Mishra, 2007).

The results of experiment 2 are in agreement with the data reported by Mathlouthi *et al.* (2002), who noticed a significant increase of nutrient digestibility and of the apparent metabolisable energy, when xylanase and β-glucanase were used in rice-based broiler diets. Rutherfurd *et al.* (2007) also showed that the enzyme supplements to corn-soybean based diets, increased the ileal digestibility of amino acids in broilers, which was 2-3% higher for methionine. Mushtaq *et al.* (2009) reported an improvement of the apparent metabolisable energy and digestibility coefficient of nitrogen when enzyme supplementation was used in diets with 30% cottonseed meal but failed to show any improvement in performance of birds fed on cottonseed meal-based diets.

The mechanisms whereby exogenous enzymes improve apparent amino acid digestibility coefficients are complex. Reducing gut viscosity and increasing cell wall permeability, xylanase facilitates the digestion of protein/amino acids (Selle *et al.*, 2009). The recent finding of Moss *et al.* (2018) showed that enzyme supplementation of broilers diets containing canola meal, a plant from the same family as camelina, significantly increased the digestibility of 9 essential amino acids and 7 nonessential amino acids. Moreover, better responses were recorded in apparent ileal digestibilities of phosphorus, calcium, sodium, zinc and five other trace minerals. In spite of many studies which reported the effect of enzymes supplementation of diets on animal performance and nutrients digestibility, information is lacking on the effect of supplementing PUFA enriched diets with exogenous enzymes and copper, on amino acid digestibility.

In conclusion, supplementation of diets enriched in PUFA Ω 3 with exogenous enzyme (0.0125%) and Cu (150 mg/kg), can alleviate the losses in apparent fecal digestibility of amino acids resulted from adding flaxseed meal and camelina meal in diets. Considering the lack of studies regarding the synergistic actions of copper and exogenous enzymes on amino acid digestibility from PUFA enriched diets, further researches are necessary.

ACKNOWLEDGEMENTS

This work was funded by the Romanian Ministry of Research and Innovation through Program 1 – Developement National Research-Developement, Sub-program 1.2 – Institutional Performance - Projects funding excellence in R & D, Contract no.17 PFE/ 17.10.2018, and project number 8PCCDI/2018 PC3, within PN-III-P1-1.2-PCCDI-2017.

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