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Timing carbon turnover ($\delta^{13}C$) in weaned piglet's brain by IRMS

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ABSTRACT

Isotope-ratio mass spectrometry (IRMS) is a potential tool that provides time-integrated estimate of assimilated and not just ingested nutrients. This feature turned possible its application to evaluate the effects of dietary nucleotides and glutamate on carbon turnover ($\delta^{13}C$) in the brain of weanling piglets. Eighty-seven piglets weaned at 21-day-old were used, being three piglets slaughtered at day-0 (prior to diet-switch) and, the remaining 84 randomly assigned in a complete block design with a 2 x 2 factorial arrangement of treatments (two nucleotide levels: 0% and 0.1% and, two glutamate levels: 0% and 1%). On trial days 3, 6, 9, 14, 21, 35 and 49, three piglets per treatment were also slaughtered. The samples were analyzed by IRMS and adjusted to the first-order equation by a nonlinear regression analysis using NLIN procedure of SAS, in order to obtain the exponential graphics. The carbon turnover ($t_{95\%}$) verified for cerebral tissue was faster ($P<0.05$) for diet containing glutamate in comparison to other diets, supporting the fact that glutamate contributed to develop the piglets' brain, due to the fastest incorporation of ^{13}C-atoms in this tissue at post-weaning phase, despite the energy deficit experienced by them.

Key words: additives, amino acids, isotopic dilution, stable isotopes, swine.

INTRODUCTION

In the last decades, the stable isotopes have been used in a growing and continuous way in agricultural sciences as a promising tool to study processes related to the digestion, absorption and metabolism of nutrients in humans and animals, as well as for the purposes of meat traceability and animal's nutrient-based dietary patterns (Gannes et al. 1998). This technique is particularly suitable for determining the relative contribution of two isotopically distinct sources of organic matter in animal diet, providing information about the integration time of the assimilated food and not only of the ingested food (Tieszen et al. 1983), representing about 50% of chemical elements that constitute animals' body (Rennie 1999)

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and, highlighting its potential for nondestructive sampling, as well as alternative potential to radioactive's isotope usage (^{3}H- thymidine).

Body tissues reflect the isotopic signal of diets that animals consume; thus, the natural variation analysis of stable isotopes abundance represents a powerful tool for the researchers, in case of a possible replacement of diet containing animal-ingredients by plant-ingredients or vice versa. In general, fast-turnover tissues will reflect more recent diets, because they will be more metabolically active, whereas those with slower turnover rates will reflect diets from old periods and therefore, will be less metabolically active (Hobson and Clark 1992). In this context, the study of organs' turnover is fundamental to determine how the isotopic signature of an animal's diet is incorporated and to demonstrate how the turnover of a tissue is related to its metabolic rate (Thompson et al. 1998).

As early-weaning of piglets causes stress characterized by a decrease in feed intake followed by changes in growth rates, a fast recovery represents an essential step for proper growth of these animals. Besides that, post-weaning is a critical step for piglets, their requirement for nucleotides is higher; however, its percursors' availability (energy and glutamine) are at low levels. Previous studies have assessed a potential role for dietary glutamic acid and nucleotides as performance-enhancing additives (Lackeyram et al. 2001, Rezaei et al. 2013, Weaver and Kim 2014). Therefore, the dietary supplementation of glutamate and nucleotides could present an additive-enhancing action and/or a health improvement for weanling piglets; although, information regarding these additives with young animals is scarce, as well as, the studies carried out with pigs at this phase have been hard to unveil due to technical limitations.

Glutamate is a multifunctional amino acid that acts as an essential substrate of intermediate metabolism, being important especially for skeletal muscle, brain, kidneys and liver (serves as a major energy substrate) (Novak et al. 1994, Yamamoto et al. 1997, Newsholme et al. 2003). Besides that, it activates taste receptors in stomach (Brosnan 2000) and, it is classified as an excitatory neurotransmitter (Liu et al. 2002). On the other hand, nucleotides are important in providing nitrogen bases and nucleosides for tissues deficient on nucleotide synthesis. They have also shown to benefit mitotic rates (Sauer et al. 2011) and the digestive tract development (Giacometti 1979, Domeneghini et al. 2004, Amorim et al. 2017). Considering the aforementioned, this study aimed at evaluating the influence of dietary glutamic acid and nucleotides on carbon turnover (δ^{13}C) in piglets' brain using isotope-ratio mass spectrometry (IRMS) as a tool for isotopic characterization of diets and cerebral tissue.

MATERIALS AND METHODS

The experiment was carried out at swine production area at Faculty of Veterinary Medicine and Animal Science from São Paulo State University (UNESP), Botucatu Campus with 87 piglets of a crossbred commercial lineage (Landrace x Large White) weaned at 21 days of age and an average weight of 6.30 ± 0.13 kg. All experimental procedures were previously approved by the Animal Ethics Committee of São Paulo State University (UNESP) under protocol number 141/2014 and, in accordance with directive 2010/63/EU.

The 84 animals (one piglet per pen with different weight categories: light, medium and heavy) were housed in a nursery facility with a ceiling height of 3.5 m and lateral curtains and, suspended in metal pens with an area of 1.0 m x 1.75 m, equipped with one feeder (with three compartments for feeding), one nipple-type drinker and, one heater. The pens had a partially slatted plastic flooring, and a compact concrete floor under the heater. A water layer under the pens was replaced once or twice a week depending on volume of waste produced.

The internal temperature of nursery facility was controlled by adjustment of lateral curtains and management of heaters.

Piglets were fed *ad libitum* within a phase feeding program to attend its nutritional requirements, in accordance with Rostagno et al. (2011), as follows: pre-starter I (21 to 35 days), pre-starter II (36 to 50 days) and starter diets (51 to 70 days). The evaluated treatments were additive-free diet: control (C); diet containing 0.1% nucleotides (Nu) (PubChem CID: 21712, 20819); diet containing 1% glutamic acid (GA) (PubChem CID: 33032) and, diet containing the mixture of 0.1% Nu and 1% GA (Nu+GA) presented in Table I.

The source of nucleotides (98% of chemical purity) was constituted by disodium guanylate and disodium inosinate. The main energy source of these diets was rice grits, a raw ingredient coming from the C_3-photosynthetic cycle, which showed a ^{13}C-isotopic signal distinct from diets provided to sows, because gestation and lactation diets primarily contained corn (a C_4-photosynthetic cycle), as energy source.

At baseline (day-0), three piglets were slaughtered, in order to express the isotopic composition of the tissue, which was a function of diets provided to sows in farrowing phase. The remaining piglets (84) were randomly allotted to

TABLE I
Percentual composition and calculated nutritional values of experimental diets.

Ingredients	Pre-starter I diets (%)				Pre-starter II diets (%)				Starter diets (%)			
	C[1]	Nu[1]	GA[1]	Nu+GA[1]	C[1]	Nu[1]	GA[1]	Nu+GA[1]	C[1]	Nu[1]	GA[1]	Nu+GA[1]
Rice grits	53.44	53.34	52.44	52.34	59.19	59.09	58.19	58.09	65.28	65.18	64.28	64.18
Soybean meal 45%	20.00	20.00	20.00	20.00	24.00	24.00	24.00	24.00	27.50	27.50	27.50	27.50
WPC[2]	6.50	6.50	6.50	6.50	3.00	3.00	3.00	3.00	-	-	-	-
Sugar	4.00	4.00	4.00	4.00	1.00	1.00	1.00	1.00	-	-	-	-
Maltodextrin	4.00	4.00	4.00	4.00	2.68	2.68	2.68	2.68	-	-	-	-
SPC[3]	2.51	2.51	2.51	2.51	2.00	2.00	2.00	2.00	1.00	1.00	1.00	1.00
Corn gluten meal	2.60	2.60	2.60	2.60	2.00	2.00	2.00	2.00	1.00	1.00	1.00	1.00
Soybean-oil	1.30	1.30	1.30	1.30	1.60	1.60	1.60	1.60	1.40	1.40	1.40	1.40
Dicalcium phosphate	1.85	1.85	1.85	1.85	1.77	1.77	1.77	1.77	1.65	1.65	1.65	1.65
Limestone	0.74	0.74	0.74	0.74	0.74	0.74	0.74	0.74	0.74	0.74	0.74	0.74
NaCl	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
L-Lys.HCl (78%)	0.82	0.82	0.82	0.82	0.58	0.58	0.58	0.58	0.33	0.33	0.33	0.33
DL-Met (99%)	0.29	0.29	0.29	0.29	0.20	0.20	0.20	0.20	0.08	0.08	0.08	0.08
L-Thr (98%)	0.34	0.34	0.34	0.34	0.23	0.23	0.23	0.23	0.14	0.14	0.14	0.14
L-Trp (99%)	0.05	0.05	0.05	0.05	0.03	0.03	0.03	0.03	0.00	0.00	0.00	0.00
L-Iso (99%)	0.14	0.14	0.14	0.14	0.02	0.02	0.02	0.02	-	-	-	-
L-Val (96%)	0.22	0.22	0.22	0.22	0.08	0.08	0.08	0.08	-	-	-	-
ZnO (73%)	0.34	0.34	0.34	0.34	-	-	-	-	-	-	-	-
Choline chloride	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09

TABLE I (continuation)

Ingredients	Pre-starter I diets (%)				Pre-starter II diets (%)				Starter diets (%)			
	C[1]	Nu[1]	GA[1]	Nu+GA[1]	C[1]	Nu[1]	GA[1]	Nu+GA[1]	C[1]	Nu[1]	GA[1]	Nu+GA[1]
Sweetener[4]	-	-	-	-	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
B.H.T.[5]	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Mineral premix[6]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin premix[7]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Glutamic acid (98.5%)	-	-	1.00	1.00	-	-	1.00	1.00	-	-	1.00	1.00
Nucleotides[8] (97%)	-	0.10	-	0.10	-	0.10	-	0.10	-	0.10	-	0.10
Isotopic values ($\delta^{13}C_{V\text{-}PDB,}$ ‰)	-26.49	-26.81	-26.50	-26.51	-27.49	-27.73	-27.05	-27.44	-28.77	-29.03	-28.65	-28.77
Calculated nutritional values												
ME (kcal/kg)	3,407	3,407	3,407	3,407	3,401	3,401	3,401	3,401	3,363	3,363	3,363	3,363
CP (%)	19.00	19.00	19.00	19.00	19.62	19.62	19.62	19.62	19.67	19.67	19.67	19.67
Dig. Lys (%)	1.46	1.46	1.46	1.46	1.33	1.33	1.33	1.33	1.17	1.17	1.17	1.17
Dig. Met (%)	0.57	0.57	0.57	0.57	0.49	0.49	0.49	0.49	0.38	0.38	0.38	0.38
Dig. Thr (%)	0.91	0.91	0.91	0.91	0.84	0.84	0.84	0.84	0.76	0.76	0.76	0.76
Dig. Trp (%)	0.25	0.25	0.25	0.25	0.24	0.24	0.24	0.24	0.23	0.23	0.23	0.23
Dig. Met+Cys (%)	0.82	0.82	0.82	0.82	0.76	0.76	0.76	0.76	0.65	0.65	0.65	0.65
Dig. Ile (%)	0.86	0.86	0.86	0.86	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78
Dig. Leu (%)	1.41	1.41	1.41	1.41	1.46	1.46	1.46	1.46	1.43	1.43	1.43	1.43
Dig. Val (%)	1.00	1.00	1.00	1.00	0.91	0.91	0.91	0.91	0.85	0.85	0.85	0.85
LE (%)	10.15	10.15	10.15	10.15	5.01	5.01	5.01	5.01	-	-	-	-
Ca (%)	0.86	0.86	0.86	0.86	0.82	0.82	0.82	0.82	0.78	0.78	0.78	0.78
Disp. P (%)	0.45	0.45	0.45	0.45	0.42	0.42	0.42	0.42	0.38	0.38	0.38	0.38

[1]C = control diet, Nu = nucleotides diet, GA = glutamic acid diet, Nu+GA = nucleotides + glutamic acid diet; nutritional value of ingredients proposed by Rostagno et al. (2011); [2]WPC = whey protein concentrate; [3]SPC = soy protein concentrate; [4]Composed by sodium saccharin, neohesperidin and silicon dioxide; [5]B.H.T. = Butylated hydroxytoluene antioxidant; [6]Mineral premix (supplied per kg of diet): Fe = 100 mg; Cu = 10 mg; Mn = 40 mg; Zn = 100 mg; Co = 1 mg; I = 1,5 mg; [7]Vitamin premix (supplied per kg of diet): Vit. A = 15,000 I.U.; Vit. D_3 = 3,000 I.U.; Biotin = 0.3 mg; Niacin = 45 mg; Calcium pantothenate = 30 mg; Folic acid = 4.5 mg; Thiamine = 3 mg; Riboflavin = 9 mg; Pyridoxine = 4.5 mg; Vit. E = 75 mg; Cyanocobalamin = 45 μg; Vit. K_3 = 3 mg; Se = 0.45 mg; [8]Composed by 5'-disodium inosinate and 5'-disodium guanylate. ME = metabolizable energy; CP = crude protein; Dig. = digestible; LE = lactose-equivalent; Disp. = disponible.

a block design with 2 x 2 factorial arrangement of treatments (two levels of nucleotides: 0% and 0.1% and, two levels of glutamic acid: 0% and 1%) and at days 3, 6, 9, 14, 21, 35 and, 49 post-weaning, three piglets per treatment (one piglet from each weight category) were slaughtered after electronarcosis. Then, a 4-cm^2 area from brain was sampled, washed with de-ionized water and packed into plastic bags, labeled and immediately frozen (-18°C) for further isotopic analyses.

In order to perform the isotopic analyses, the samples were previously defrosted and dried in a circulating-air oven (model MA-035/5, Marconi, Piracicaba, Brazil) at 50°C for 48-hour. As the lipid fraction might cause isotopic fractionation up to 5‰ of ^{13}C values (Piasentier et al. 2003), the samples were degreased with ethyl ether (chemically pure) in a Soxhlet apparatus (model TE-044, Tecnal, Piracicaba, Brazil) at 65°C for 4-hour and submitted again to drying at 50°C for 30-min to removal of ethyl ether residual. Then, samples were cryogenic milled (model 2010, SPEX SamplePrep Geno/Grinder, Metuchen, USA) as follows: diets and ingredients: 8 cycles of 6-min at 1,000 rpm, brain: 1 cycle of 6-min at 990 rpm under -190°C, in order to obtain homogeneous material (particle size <60 μm) and to avoid repeatability problems at analyses' step.

The samples (50 to 70 μg) were weighted in an analytical micro balance (model XP_{26}, Mettler Toledo, Columbus, USA) into tin capsules, which were then introduced by means of an automatic sampler (model AS-3000, Thermo Fisher Scientific, Waltham, USA) in an elemental analyzer (model Flash CHNS/O 2000, Thermo Fisher Scientific, Waltham, USA) where, under the presence of oxygen, each sample was quantitatively combusted to obtain CO_2 following the isotopic gas combinations and their respective m/z: $^{12}C + ^{16}O + ^{16}O = 44$; $^{13}C + ^{16}O + ^{16}O = 45$, and $^{13}C + ^{16}O + ^{16}O = 46$. The generated gases were separated on a gas chromatographic column (PN 26008205, Thermo Scientific, Germany) maintained at 45°C and analyzed on an isotope ratio mass spectrometer (IRMS - Delta V Advantage, Thermo Fisher Scientific, Waltham, USA) interconnected to the software Isodat 3.0 (Isomass Scientific, Calgary, Canada) at the Center of Environmental Stable Isotopes, Biosciences Institute of UNESP, Botucatu Campus.

The mass spectrometer calibration was performed with CO_2 6.0 (99.99% of chemical purity) from a cylinder, which was previously calibrated against the official reference material IVA-33802174 (certificate number: 147273, Meerbusch, Germany), consisting of a certified urea, which provided a value of $\delta^{13}C_{V\text{-}PDB} = -39.73‰ \pm 0.2‰$. This certified urea was used for a 6-month period to ensure the spectrometer calibration. The $^{13}C/^{12}C$ standard isotope ratio was expressed as δ (delta) ‰ (per thousand) relative to the PDB (Pee Dee Belemnite), the primary standard. At the beginning of each run, two pulses of CO_2 reference gas were admitted in the system for about 5 s. The constant flow rate during this period has given peaks a flat-top appearance. A level of CO_2 corresponding to 5 V at m/z 44 was used to calibrate the system.

For internal quality control of analyses, during each cycle of readings (at the beginning and the end of each 45-sample sequential analyses), an internal standard was used to ensure the accuracy of quantifications. The internal standard was a coal from eucalyptus-plant, calibrated and analyzed against the working standard (IVA). The averaged $\delta^{13}C$-signal obtained after triplicate analyses of the coal was -28.00‰ ± 0.2‰. Samples were analyzed in duplicate and, standard analyzed in four replicates and then, the values averaged. The precision of method was set at 12‰ that refers to the amplitude of isotopic signals between C_3- and C_4-plants. The certainty of method was set as 0.2‰ of standard deviation.

The spectrometer readings were expressed as $\delta^{13}C_{V\text{-}PDB}$ (‰) notation in relation to the PDB

international standard with analysis deviation at the order of 0.2‰, calculated by the equation:

$\delta^{13}C_{(sample,\ standard)} = \{[(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}] / (^{13}C/^{12}C)_{standard}\} \times 10^3$ that can be summarized with the following equation: $\delta^{13}C_{(sample,\ standard)} = [(R_{sample} / R_{standard}) - 1] \times 10^3$, where: $\delta^{13}C$ = the enrichment of the isotopic ratio $^{13}C/^{12}C$ of the sample to the standard (‰); R = the ratio of the heavier (^{13}C) to the lighter (^{12}C) stable isotopes (dimensionless).

To evaluate the carbon substitution rate of samples, the following exponential function over time was employed (Ducatti et al. 2002): $\delta^{13}C_{(t)} = \delta^{13}C_{(f)} + (\delta^{13}C_{(i)} - \delta^{13}C_{(f)})\ e^{-kt}$, where: $\delta^{13}C_{(t)}$ = isotopic enrichment of tissue at any time (t); $\delta^{13}C_{(f)}$ = isotopic enrichment of tissue at the equilibrium or final condition; $\delta^{13}C_{(i)}$ = isotopic enrichment of tissue at the beginning; k = turnover constant (days), and t = time (days) since diet switch (C_4- to C_3-based diet).

The carbon half-life ($T_{50\%}$) of samples at t = T necessary for initial atoms substitution by the final atoms was determined by the equation (Ducatti et al. 2002): $t_{n\%} = (-1 / k) \ln (1 - F)$, where: t = time of initial atoms substitution by the final substitution (days), ln = natural logarithm, F = value of atom substitution, which can vary among 0.0 to 0.95, and k = turnover constant (days).

It means that when F = 0.50, there is 50% of carbon substitution, thus the half-life value ($T_{50\%}$) is achieved, which gives the estimated organ turnover. The half-life equation allows us to infer that tissues, which provide lower half-life values, will present a higher metabolization or incorporation of stable isotopes; the opposite being true. In contrast, when F = 0.95, there is 95% of carbon substitution ($t_{95\%}$), that is, the isotopic turnover was stabilized, so the tissue has reached its isotopic-plateau level (Ducatti et al. 2016). The calculation of the isotopic substitution plateau reflects the reliability in half-life values, in order to assess if experimental period was enough for ^{13}C-substitution in a particular tissue. By this way, researchers can use the half-life parameter to compare their treatments (Table II).

The carbon isotopic enrichment data ($\delta^{13}C$, ‰) analyzed by IRMS were compiled *versus* each slaughter day throughout 49 days. Subsequently, each set of data (slaughter day *versus* brain-$\delta^{13}C$ from each piglet) were adjusted to the first-order equation by a nonlinear regression analysis using the NLIN of SAS (SAS Institute, Cary, NC, USA) in order to establish the $\delta^{13}C$ over time. The exponential graphics were plotted by software OriginPro 8.07 (OriginLab Corporation, Northampton, MA, USA) to further calculations and comparison between half-life values ($T_{50\%}$) and $t_{95\%}$ of treatments that were subjected to analysis of variance using the GLM (general linear model) procedure of the SAS software, v.9.2 (SAS Institute Inc., Cary, NC, USA), and the means compared according to each weight category of piglets by Tukey's test at 5% of significance.

RESULTS

The diet provided to lactating sows, as well as, to suckling piglets reflected the isotopic signature of C_4-plant, presenting a mean value of $\delta^{13}C$ = -16.15‰, due to the main ingredient of this diet was corn, a C_4-plant, which when analyzed, presented a mean value of $\delta^{13}C$ = -17.40‰. The isotopic discrimination between cereals were observed in this study, due to alteration over time

TABLE II
Half-life ($T_{50\%}$) and ^{13}C total substitution ($t_{95\%}$) values of piglets' brain according to experimental diets.

	Diets[1]			
Brain turnover (days)	**C**	**Nu**	**GA**	**Nu+GA**
$T_{50\%}$	18.97 a	20.53 b	18.40 a	18.66 a
$t_{95\%}$	82.00 C	88.71 D	79.53 A	80.63 B

C = control diet, Nu = 0.1% nucleotides diet, GA = 1% glutamic acid diet, Nu+GA = 0.1% nucleotides + 1% glutamic acid diet. Average values in the same row followed by different lowercase / uppercase letters differ (P<0.05) by Tukey's test.

of diet's isotopic signature from corn to rice, which has given diet mean values of $\delta^{13}C_{V\text{-}PDB}$, as follow: -27.59‰, -27.86‰, -27.40‰ and, -27.57‰ in C-diets; Nu-diets; GA-diets and, Nu+GA-diets, respectively.

The brain of piglets fed Nu-diet presented the slowest carbon turnover value ($P<0.05$) (Table II), due to 81% of carbon-13 substitution within 49 days, resulting on higher half-life ($T_{50\%}$) and 95% of ^{13}C-substitution ($t_{95\%}$) values. Besides that, cerebral tissue of piglets fed C-diet needed almost 7-day less than those fed the Nu-diet to incorporate 83% of carbon-13 atoms during the same period (2% more carbon-13 atoms substituted than Nu-diet), which has favored the reduction ($P<0.05$) of its $t_{95\%}$-value.

However, piglets fed Nu+GA-diet had 84% of carbon-13 atoms incorporation in their brain, providing $T_{50\%}$-value of 7.44-h faster than piglets fed C-diet and 1-d and 22-h faster than animals fed Nu-diet. In addition, the Nu+GA-diet determined the second lower $t_{95\%}$-value ($P<0.05$) compared to other diets, which probably occurred due to the presence of glutamate, because piglets fed GA-diet presented the fastest ($P<0.05$) brain turnover ($t_{95\%}$) in relation to piglets fed other diets, so that carbon-13 atoms incorporation during experimental period was 84%, culminating in $T_{50\%}$- and $t_{95\%}$-values, respectively, 14-h and 2.5-d faster than those verified for C-diet.

Regarding isotopic composition of brain, Figure 1 shows that trial duration was not sufficient for cerebral tissue to reach the isotopic plateau, because the obtained $t_{95\%}$-values were higher than 49 days (Table II). Despite this, the experimental period was sufficient for the isotopic dilution's occurrence in this organ, because $\delta^{13}C_{V\text{-}PDB}$ values of piglet's brain on day-0 reflected isotopic signals averaged -15.83‰, characteristic signal of corn (C_4-plant: -16.15‰) and, due to $\delta^{13}C_{V\text{-}PDB}$ value verified in this tissue at the end of experiment (day-49) on average was -23.86‰, characteristic signal of rice (C_3-plant: -30.14‰) provided to piglets of 21- to 70-day-old.

DISCUSSION

The results of experimental diets (Table I) demonstrated that isotopic signals were close to $\delta^{13}C$ = -27‰, as expected, because they reflected the isotopic signature of rice, indicating the isotopic alteration as a function of diets provided to piglets (Smith and Epstein 1971), as well as the isotopic discrimination between cereals (corn *versus* rice) (Park and Epstein 1961), allowing us to state that principles of isotopic dilution (Hobson and Clark 1992, Jones et al. 1979, Tieszen et al. 1983) were achieved in this experiment and also that isotopic composition of diets can be estimated by carbon-13 isotopic value of piglet's brain. The obtained results corroborate those reported by DeNiro and Epstein (1978) that verified the organic matter analysis of diet results in a more reliable measurement of isotopic data ($\delta^{13}C$, ‰) in relation to other biochemical fractions, as the lipid, carbohydrate and the protein.

The role of glutamate in cerebral metabolism is very important because it is the major excitatory neurotransmitter in the central nervous system of mammals and it is the precursor of γ-aminobutyric acid (GABA), a predominantly inhibitory neurotransmitter (Mangia et al. 2012). Also, it is the only amino acid oxidized by the brain, because the enzyme glutamate-deaminase acts by catalyzing the reverse reaction (the production of glutamate from ammonia and α-ketoglutarate) that in turn, accelerates the carbohydrate metabolism (Weil-Malherbe 1936), demonstrating the anaplerotic action of glutamate in Krebs' cycle.

In addition, the study of Nachmansohn et al. (1943) reported that dietary L-glutamate supplementation increased 4 to 5 times acetylcholine synthesis, demonstrating its key role in brain function. Other study verified the role of

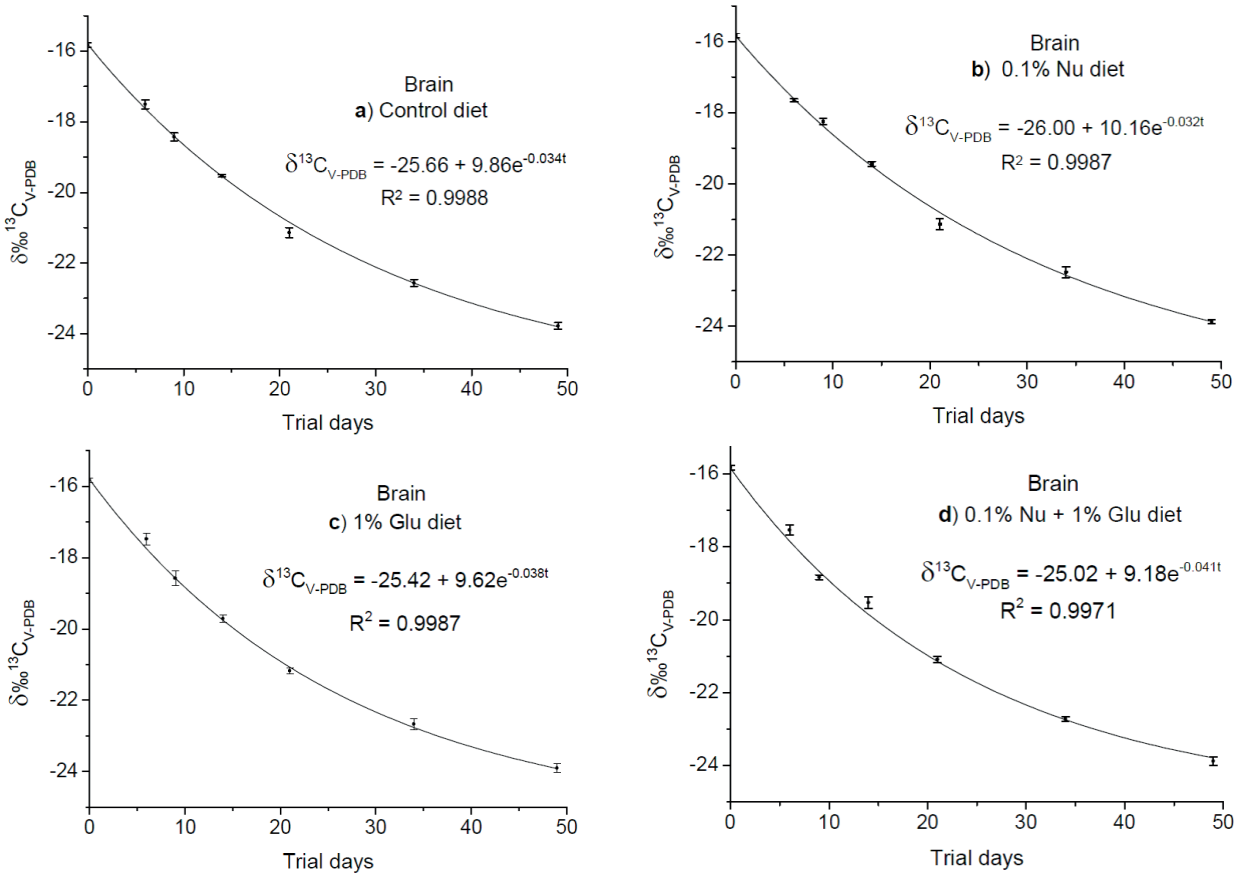

Figure 1 - Non-linear adjustment of exponential curves ($\delta‰^{13}C_{V\text{-}PDB}$) of piglets' brain (**a**): control diet, (**b**): 0.1% nucleotides, (**c**): 1% glutamic acid, and (**d**): 0.1% nucleotides + 1% glutamic acid. Values expressed as: mean (n = 3) ± standard error.

glutamic acid on increased learning of rats fed diets containing L-glutamate in relation to those fed a control diet, as well as the increased percentage of rats able to overcome obstacles to access the diet (Zimmerman and Ross 1944), and increased intelligence score of children with intellectual disability who consumed 12 g day^{-1} of L-glutamate for 6 months that have showed significant improvements in verbal and motor performance tests, as well as changes in traits of their personality (Zimmerman et al. 1947).

Gibbs et al. (2007) demonstrated that glycogen is the preferred precursor of glutamate by nervous cells, the astrocytes (through α-ketoglutarate transamination) and neurons (through glutamine hydrolysis) of 1-day-old chicks and, that glutamate has been involved since the early days of life in the learning process of birds. In addition, a research with early-weaned piglets supplemented with 6.51% L-glutamate of analytical purity during 12 days reported an improvement in total growth of small intestine, as well as in duodenal mucosa, preventing atrophy of duodenum villi that normally occurs after weaning compared to piglets fed a control diet or a diet supplemented with polyamines (Ewtushik et al. 2000).

Moreover, considering the affinity of glutamate for brain tissue and the fact that several studies have demonstrated its importance, it is possible that in the present study, its supplementation may have contributed to the brain growth (protein synthesis) (Platt 2007) due to the fastest turnover

($t_{95\%}$) observed, because glutamate is the only amino acid capable of serving as a source of oxoglutarate to maintain brain activity regardless of glucose levels (Weil-Malherbe 1936, Woodman and McIlwain 1961), it has post-synaptic action (Tapiero et al. 2002) and it is oxidized by the brain to release α-ketoglutarate (for glucose synthesis through Krebs' cycle) and ammonia (through transdeamination) (Nelson and Cox 2012).

In conclusion, the supplementation of 1% glutamic acid was efficient to develop the brain of piglets, despite the energy deficit experienced by them at post-weaning period. However, further research is needed to evaluate the economic feasibility of its inclusion in commercial diets and its most adequate supply period. To date, IRMS for timing carbon turnover ($\delta^{13}C$) is an important methodology in animal nutrition' studies (e.g. obtaining biomolecules as biomarkers or as substitute for radioisotopes' usage).

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